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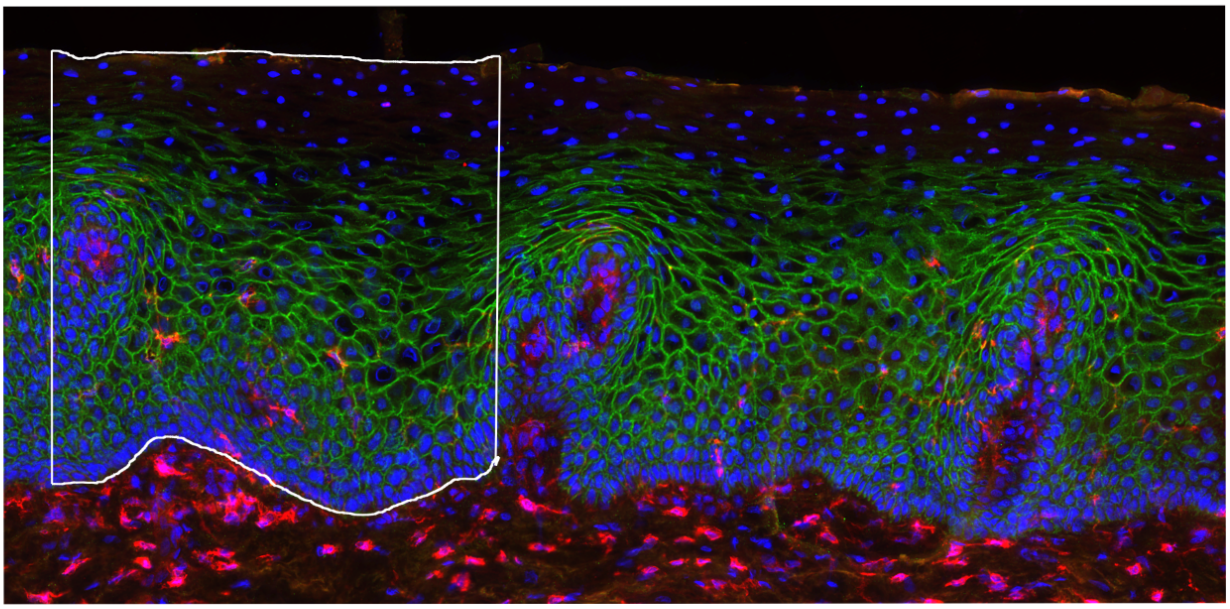
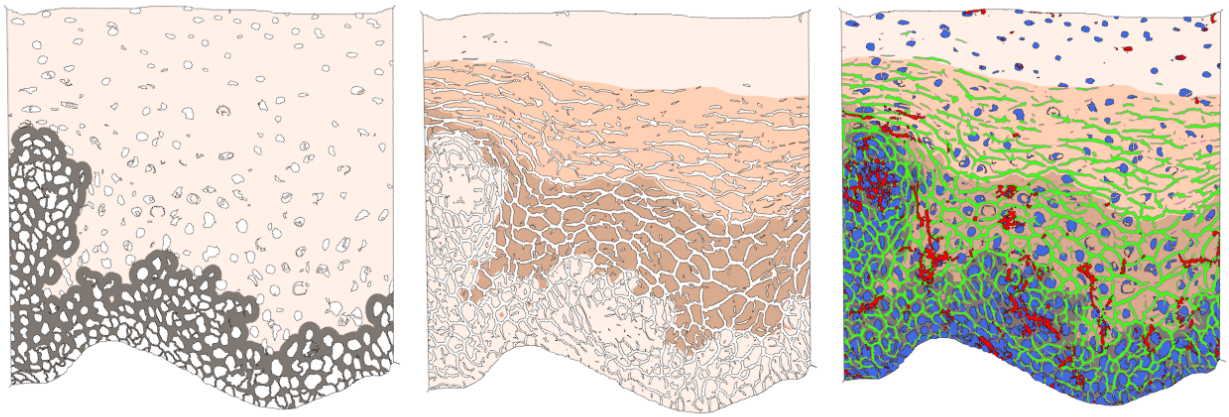
Epithelial barrier protection - implications for HIV susceptibility





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Epithelial barrier protection
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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To all women breaking barriers

"The real voyage of discovery
consists not in seeking new landscapes
but in having new eyes"

Marcel Proust

POPULÄRVETENSKAPLIG SAMMANFATTNING

Majoriteten av nya hiv-infektioner sker via sexuell överföring där hiv-viruset måste ta sig igenom mottagarens skyddande genitala slemhinna för att träffa på en målcell att infektera. I denna avhandling har vi studerat vävnadsprover från den genitala och rektala slemhinnan, och hur den påverkas av olika faktorer. Slemhinnan består av epitelceller som hålls samman av ett ”klisterprotein”, E-cadherin. Inuti slemhinnan finns olika immunceller bl.a $CD4^+$ hiv målceller och $CD8^+$ T-celler som kan döda virus-infekterade celler. Genom att fotografera histologiskt infärgade vävnads-snitt från apa och människa har vi visualiserat olika celler och strukturen av E-cadherin. Vi har utvecklat digitala bildanalysprogram för att automatiskt mäta hur epitelet hålls samman samt kvantifiera antalet immunceller.

I arbete **I** undersökte vi ifall en profylaktisk gel som blockerar hiv från att binda till målceller, hade negativ påverkan på den rektala slemhinnan hos apor. Med hjälp av bildanalys såg vi att epitelet var opåverkat men att behandlingen ökade antalet $CD4^+$ målceller i slemhinnan marginellt vilket inte tros öka risken för hiv vid användande av gelen.

I arbete **II** studerade vi en vävnadsspecifik typ av $CD8^+$ minnes-T-celler. Vaccin som stimulerar tillverkning av vävnadsspecifika minnes-celler ger ett starkt skydd mot infektioner. Vi visade för första gången att dessa vävnadsspecifika minnes-celler fanns i genital-slemhinnan hos kvinnor, samt att hiv-infekterade kvinnor hade fler av dessa celler som inte uttryckte CD103, ett protein som binder till E-cadherin och därmed ankrar fast cellerna i slemhinnan. Vår data tyder på att hiv-infekterade kvinnor har ett större inflöde av dessa $CD8^+$ minnes-T-celler till slemhinnan, men att dessa celler ej har hunnit uppreglera sina CD103 proteiner.

Kvinnor som använder en viss typ av p-spruta som innehåller ett konstgjort progesterone (DMPA) har visat sig ha ökad risk att smittas av HIV. I arbete **III** visade vi att kvinnor som använder DMPA hade ett tunnare ytskikt på sin slemhinna och att deras målceller låg närmare ytan. Detta tyder på att hiv enklare kan passera slemhinnan och snabbare träffa på en målcell, vilket kan förklara den ökade hiv-risken vid DMPA-användning.

Slemhinnan är täckt av bakterier, goda bakterier kan stärka slemhinnans försvar medan elakartade bakterier kan bidra till ökad hiv risk. I arbete **IV** upptäckte vi att kvinnor med en viss typ av Lactobaciller hade en stabilare slemhinna. Vi mätte även proteiner i sekret från

slemhinnan och såg att de kvinnor som hade de goda bakterierna Lactobaciller var kopplade till högre nivåer av anti-inflammatoriska och epitel-stabiliserande proteiner.

Sammantaget visar denna avhandling att bildanalys är ett användbart verktyg för att studera den genitala slemhinnan. Olika faktorer påverkar stabilitet och närvaro av immunceller i slemhinnan vilket kan leda till ökad hiv risk. Dessa resultat lägger grunden för framtida forskning att utveckla metoder för att stärka slemhinnan och öka skyddet mot sexuellt överförbara infektioner.

ABSTRACT

The majority of HIV infections today occur through sexual HIV transmission. The female genital mucosa offers a barrier against incoming pathogens. Although, studies show that the vaginal microbiome, co-current infections and local inflammation, the use of hormonal contraceptives and microbicides, can weaken this protective lining. In this thesis *in situ* digital image analysis workflows were developed and used together with protein profiling, to characterize the effects of such factors on the genital mucosal barrier integrity and the immune cells therein.

Topically applied microbicides can protect against HIV. In paper I we introduced image analysis as a refined tool for evaluation of microbicide safety. We confirmed that a promising microbicide candidate, Q-GRFT, had no negative effect on the rectal epithelium while causing a small, but probably biologically negligible, increase in $CD4^+$ HIV target cells. We also discovered potential effects of multiple biopsy sampling that should be considered when designing pre-clinical studies.

Specific tissue resident immune cells with an effector memory phenotype have a rapid response against re-infections and may be important against HIV infection. In paper II, we observed that HIV infected women had increased levels of $CD103^-CD8^+$ tissue resident memory cells compared to uninfected women, and that this may be due to a recent influx of these effector cells that have not yet upregulated the CD103 retention molecule.

In paper III we revealed that women taking the hormonal contraceptive DMPA, had a thinner superficial layer of the female genital mucosa. Lack of this protective layer in combination with having more HIV target cells located closer to the vaginal lumen, could contribute to the increased HIV risk in women taking DMPA.

In paper IV we discovered that *Lactobacillus non-iners* dominated women had a more intact epithelium, and *Gardnerella* dominated women had a different spatial localization of $CD4^+$ cells in the epithelium. Secreted protein profiles from *Lactobacillus* dominant women had elevated levels of anti-inflammatory and epithelial barrier proteins compared to non-*Lactobacillus* dominant women. These factors may contribute to reduced HIV risk in *Lactobacillus*-dominant women.

The results of this thesis highlight the benefits of using digital image analysis as a tool for studying spatial and structural changes in the mucosal tissue barrier and the immune cell landscape therein. We showed potential mechanisms in how different factors increase the HIV risk. These findings will support development of interventions aimed to strengthen the mucosal barrier, and thereby reduce transmission of sexually transmitted infections.

LIST OF SCIENTIFIC PAPERS

I. Impact of Q-Griffithsin anti-HIV microbicide gel in non-human primates: *In situ* analyses of epithelial and immune cell markers in rectal mucosa.
Gökçe Günaydın, **Gabriella Edfeldt***, David A. Garber, Muhammad Asghar, Laura Noèl-Romas, Adam Burgener, Carolina Wählby, Lin Wang, Lisa C. Rohan, Patricia Guenthner, James Mitchell, Nobuyuki Matoba, Janet M. McNicholl, Kenneth E. Palmer, Annelie Tjernlund and Kristina Broliden.*
Scientific Reports. 2019 Dec 2; 9:18120. *These authors contributed equally.

II. HIV-infected women have high numbers of CD103-CD8+ T cells residing close to the basal membrane of the ectocervical epithelium.
*Anna Gibbs, Marcus Buggert, **Gabriella Edfeldt**, Petter Ranefall, Andrea Introini, Stanley Cheuk, Elisa Martini, Liv Eidsmo, Terry B. Ball, Joshua Kimani, Rupert Kaul, Annika C. Karlsson, Carolina Wählby, Kristina Broliden and Annelie Tjernlund.*
The Journal of Infectious Diseases. 2018 Aug 1; 218:453-65**.**

III. Digital tissue image analysis reveals apical distribution of HIV target cells in the ectocervical epithelium of women using depot medroxyprogesterone acetate.
***Gabriella Edfeldt**, Julie Lajoie, Maria Röhl, Kenneth Omollo, Mathias Mack, Joshua Kimani, Julius Oyugi, Carolina Wählby, Keith R. Fowke, Kristina Broliden and Annelie Tjernlund.*
Manuscript.

IV. Cervicovaginal microbiota affects the human ectocervical epithelial barrier structure as determined by *in situ* digital image analysis and protein profiling.
***Gabriella Edfeldt**, Frideborg Bradley, Sofia Bergström, Julie Lajoie, Jiawu Xu, Vilde Kaldhusdal, Alexandra Åhlberg, Behnaz K.H. Binizy, Cecilia Mattsson, Anna Månberg, Carolina Wählby, Joshua Kimani, Julius Oyugi, Peter Nilsson, Keith R. Fowke, Annelie Tjernlund, Douglas S. Kwon and Kristina Broliden.*
Manuscript.

Publication not included in the thesis

Increased Cervical $CD4^+CCR5^+$ T Cells Among Kenyan Sex Working Women Using Depot Medroxyprogesterone Acetate.
Julie Lajoie, Annelie Tjernlund, Kenneth Omollo, ***Gabriella Edfeldt****, Maria Röhl, Geneviève Boily-Larouche, Julianna Cheruiyot, Makubo Kimani, Joshua Kimani, Julius Oyugi, Kristina Broliden and Keith R. Fowke.*
AIDS Research and Human Retroviruses. 2019 Mar 5; 10.1089.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral treatment
BV	Bacterial vaginosis
BVAB	Bacterial vaginosis associated bacterium
CCR	CC chemokine receptor
CD	Cluster of differentiation
CDC	Center for Disease Control and Prevention
CSTA	Cystatin A
CSTB	Cystatin B
CT	Cervicotype
CVL	Cervicovaginal lavage
CXCR	CXC chemokine receptor
DC	Dendritic cell
DMPA	Depot medroxyprogesterone acetate
FDA	Food and drug administration
FGT	Female genital tract
FSW	Female sex worker
gp120	glycoprotein 120
GR	Glucocorticoid receptor
HBD	Human beta-defensins
HIV	Human Immunodeficiency Virus
HLA-DR	Human Leukocyte Antigen - DR isotype
HPV	Human papilloma virus
HSV	Herpes simplex virus
IM	Intermediate layer
IP-10	Interferon-gamma-inducible protein 10
ITIH2	Inter-alpha-trypsin inhibitor heavy chain 2
JAMs	Junctional adhesion molecules
KRT1	Keratin 1
LC	Langerhans cell
lme	Linear mixed effect
LP	Lamina propria
MPA	Medroxyprogesterone acetate
N9	Nonoxynol-9
NHP	Non-human primate

NIH	National Institute of Health
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
OCT	Optimal cutting temperature
PBMC	Peripheral blood mononuclear cell
PCI	Phenol-chloroform-isopropanol
PI3	Peptidase inhibitor 3
PLHIV	People living with HIV
PR	Progesterone receptor
PrEP	Pre-Exposure Prophylaxis
PREVENT	PREvention of Viral ENTry
Q-GRFT	Q-Griffithsin
RM	Rhesus Macaques
RNA	Ribonucleic acid
RVI	Rabbit vaginal irritation
S1PR1	spingosine-1-phospate receptor
SIV	Simian Immunodeficiency virus
SLPI	Secretory leukocyte protease inhibitor
SPINK5	Serine peptidase inhibitor Kazal type 5
SPRR3	Small proline rich protein 3
STI	Sexually transmitted infection
TACSTD2	Tumor associated calcium signal transducer
TCM	Central Memory T cell
TEM	Effector Memory T cell
TFV	Tenofovir
TGF	Transforming growth factor
Th 17	T helper type 17 cells
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRM	Tissue Resident Memory
UNAIDS	United Nations Programme on HIV/AIDS

1 AIM OF THESIS

The general aim of this thesis was to assess the rectal and genital mucosal barrier and to characterize how different factors, such as hormonal contraceptive use, vaginal microbiome composition and microbicide applications, affect these protective linings and their local immune repertoire. The specific aims were as follows:

Paper I: To evaluate mucosal HIV susceptibility markers in a pre-clinical toxicity study of an anti-HIV gel (Q-GRFT) applied to rectal mucosa in a non-human primate model and to evaluate the use of image analysis as an *in situ* assay for safety assessment.

Paper II: To investigate if $CD8^+$ T cells residing in the ectocervical epithelium displayed a tissue-residing phenotype, and if HIV infected women had an altered phenotype of these cells.

Paper III: To assess how the hormonal contraceptive DMPA affects the barrier integrity and HIV target cells in the human ectocervical epithelium.

Paper IV: To investigate effects of the cervicovaginal microbiome composition on epithelial markers of integrity and HIV target cells *in situ*, and correlation to secreted markers of epithelial disruption and inflammation.

2 THE CURRENT HIV EPIDEMIC

2.1 IN NUMBERS

Almost 40 years have passed since the discovery of the human immunodeficiency virus (HIV) in 1983[1,2]. There are two subtypes, HIV-1 is more common than HIV-2 which is confined to West Africa, therefore HIV-1 will be referred to as HIV in this thesis. Despite being one of the most well-studied viruses, the high mutation rate of the virus, the glycan shield that hides the envelope from neutralizing antibodies and its ability to hide within latent cell reservoirs have hampered the development of both vaccine and cure. HIV is one of the most devastating sexually transmitted infections (STI) in the world, responsible for more than 32 million deaths to date[3]. Global efforts, led by the joint United Nations Programme on HIV/AIDS (UNAIDS), aim to end the HIV/acquired immunodeficiency syndrome (AIDS) epidemic by 2030, through increased testing and treatment to reduce viral loads and thereby eliminate viral spread[4,5]. The milestone for 2020 is to have 73% of people living with HIV (PLHIV) virally suppressed. Although, the most recent data from 2018 show that only 53% of the 37.9 million PLHIV had suppressed viral loads, indicating that this year's target may not be reached. 1.7 million people still get infected every year. Poverty, unequal access to care and prevention services, stigma, discrimination and sexual violence are factors that contribute to the slow decline in the number of new infections. Sub-Saharan Africa is bearing the highest burden of HIV, harboring 53% of the world's PLHIV[4,5].

2.2 HIGH RISK GROUPS

Targeting often hard-to-reach key populations is essential to achieve a sustained end to the HIV epidemic. UNAIDS estimate that 40-50% of new HIV infections occur in key populations such as people who inject drugs, men who have sex with men, transgender persons, sex workers and prisoners[4] (Figure 1). Information, improved testing and treatment is essential for these groups, although discrimination still limits access to care in many regions. Likewise, power imbalances make another group disproportionally affected by the HIV epidemic; women. More women than men are infected with HIV and for every three newly HIV infected young men (aged 15–24 years) in Eastern and Southern Africa, there are seven new infections among young women[6]. Women are more likely than men to be on antiretroviral (ART) treatment which reduces their viral loads and protects their male partners. Another biological factor increasing the HIV risk in women is that women have a larger surface area of their genital mucosa compared to men. Increased awareness about HIV as well as suitable prevention methods is key in protection of young women.

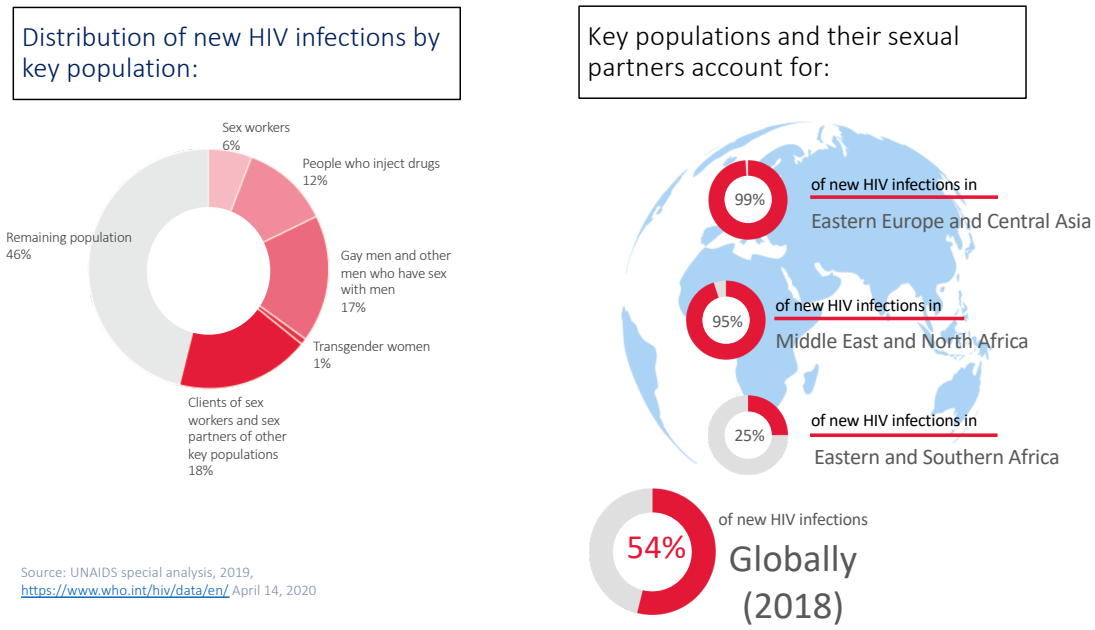

Figure 1. Distribution of new HIV infections by key population and the percentage of new infections that key populations and their sexual partners account for. To combat the HIV epidemic, it is important to target key populations. Eastern and Southern Africa have a more general spread of the infection where in particular young women are at high risk. Data from the World Health Organization, UNAIDS special analysis, 2019.

2.3 HIV TRANSMISSION

The majority of all HIV transmissions worldwide occur through sexual transmission[4]. The mucosa lining the genital and rectal tract is considered to be the main portal of entry as well as the first replication site before the virus is further spread to local lymph nodes and the circulation[7]. The HIV sexual transmission risk is influenced by a number of factors including partner viral load, virulence of the viral strain, type of sexual encounter and genital inflammation[8]. The per-act transmission is low, estimated to 0.04% for vaginal intercourse and 1.38% for receptive anal intercourse[9]. In male-to-female HIV transmission, free virions or HIV-infected cells are released with the semen in the vaginal vault. It is not yet known how the virus cross the protective epithelial barrier. It has been shown that free virions can either be picked up by protruding Langerhans cells (LC) that scan the vaginal lumen, be transcytosed through the epithelial cells or enter through breaches in the epithelium. It is further believed that components of the protein-rich seminal plasma may promote HIV infection by increasing the vaginal vault pH and cause an inflammatory rise attracting more HIV target cells[10,11].

HIV is an enveloped retrovirus belonging to *Lentivirus* genus/*Retroviridae* family, it has a single-stranded, positive-sense RNA genome that upon HIV-target cell fusion enters the host cell cytoplasm, is reverse transcribed to double-stranded DNA and incorporates into the host

cell genome, where it can hide for several years[12]. Most infections occur through binding of the HIV spike glycoprotein gp120 to the CD4 receptor, leading to subsequent binding to the CCR5 or CXCR4 co-receptor enabling membrane fusion[13]. The main HIV target cell is $CD4^+$ T cells expressing CCR5, but HIV can also infect macrophages and different dendritic cells (DC), including LC[14,15].

3 EPITHELIAL BARRIER – THE WALL OF PROTECTION

All human surfaces are covered by continuous epithelium, keratinized (skin) or non-keratinized (all other), separating the body from the external environment and offering protection, sensing, transcellular transport, secretion and selective absorption. One reason behind the low per-coital-act transmission rate of HIV is the genital epithelial barrier that efficiently protects the body against incoming pathogens. The squamous stratified epithelium covering the vagina and ectocervix is the most robust epithelium, also found on high-friction surfaces like the mouth and esophagus.

3.1 THE UNIQUE FEMALE GENITAL MUCOSA

In the human body, the dynamic cervicovaginal mucosal barrier is unique in that it is under (cyclic) hormonal influence, has a pH<4.5, is frequently exposed to inflammatory stimuli, and can balance successful implantation of a semi-foreign embryo while protect against other foreign pathogens[16,17]. The first anatomical barrier pathogens encounter is the cervicovaginal mucus, acting as a protective film on top of the epithelium. The mucus not only traps the movement of infectious particles but contains anti-microbial peptides, mucins, antiproteases, secreted from epithelial- and immune cells. These protective molecules help maintain the epithelial barrier integrity and defend against microbes[16,18]. The tightly bound cells in the epithelium form the second anatomical barrier that pathogens need to cross to infect the host. A healthy, intact epithelium has been shown to effectively stop HIV but micro-abrasions occurring during sexual intercourse, inflammation and co-current STI loosen this barrier and facilitate HIV entry[19]. The upper female genital tract (FGT) (the endocervix, uterus, fallopian tubes and ovaries) is covered by a single-layer columnar epithelium, and the lower FGT (the vagina and ectocervix), by a multilayer squamous epithelium. A thick mucus plug blocks the entrance to the endocervical canal (except during ovulation) and is suggested to hinder both semen and viruses to enter. HIV infections are therefore thought to occur more often in the lower FGT, that despite a thicker epithelium has 15 times larger surface area than the endocervix, thus offering more potential places for viral entry[19,20].

3.2 MORPHOLOGY OF THE SQUAMOS STRATITIFED EPITHELIUM

Epithelial tissue rests on a selectively permeable basement membrane where substances diffusing from the blood vessels in the underlying tissue creates a weak upward flow[21]. In the squamous stratified epithelium of the cervico-vaginal tract, there are 2-3 epithelial cell layers tightly packed close to the basement membrane, that constitutes the parabasal layer where cell division occurs. As the cells migrate up towards the lumen into the intermediate layer (stratum spinosum), they mature and their cytoplasm becomes larger, filled with glycogen. The outermost layer, the superficial layer (stratum corneum), consists of terminally differentiated cells that are flat in shape and devoid of tight junctions. Every four hours one cell layer is shed off, and the average epithelium is renewed in 96 hours[22,23]. The constant exfoliation of cells is a way of getting rid of pathogens bound to the outermost cell layers. The dead cells release their content into the lumen, a major component is glycogen which serves as substrate for *Lactobacilli*[23]. The cyclic variations in endogenous sex hormones affects the structure of the epithelium, estrogen acts on all layers promoting proliferation and desquamation while progesterone predominantly acts on the intermediate layer. During the follicular menstrual phase, when estrogen to progesterone levels is high, the superficial layer is thicker while during the luteal phase with high progesterone levels, the intermediate layer is thicker[24].

3.3 EPITHELIAL JUNCTION PROTEINS

Cell junctions work like glue between epithelial cells and have an important function in maintaining the stability of the epithelium. Tight junctions (occludins, claudins, and junctional adhesion molecules (JAMs)) control paracellular diffusion allowing ions and leukocytes to pass through, and prevents pathogens to enter[22]. Adherens junction proteins (E-cadherin) anchor in actin filaments inside the cells and keep the cells together. Desmosomes (JAM3, desmoglein and desmocollin) attached to keratin intermediate filaments create the epithelial elasticity. The parabasal cells have a strong expression of cell adhesion molecules that are diminishing when the cells become highly differentiated, and completely lost in the outermost superficial layer. The lack of cell adhesion molecules in the superficial layer render this layer easily penetrable by both pathogens and immune modulators. Experimental studies in non-human primates (NHPs) show that after inoculation the majority of HIV virions are found in the superficial layer of the ectocervix, at an average depth of about 10 μm (i.e. about one cell layer) and the maximum distance of free virus transfer recorded was 50 μm[19].

The epithelial gatekeeping proteins are plastic and respond to stimuli from estrogen, growth factors, calcium concentration, inflammatory mediators and pathogen invasion[22]. Estrogen has been shown to decrease the tight junctions occludin in cell culture and ZO-1 in human gut mucosa[21,25]. Similarly, HIV-related increase in TNF- α was shown to disrupt the tight junctions ZO-1, occludin and claudins in epithelial cell monolayers [26]. Herpes simplex virus (HSV)-2 infected men showed reduced m-RNA levels of the tight junction Claudin-1[27]. *Chlamydia trachomatis* as well as other bacterial infections have been shown to break down the adherens protein complex N-cadherin/β-catenin in cell cultures[28]. Different enzymes such as matrix metalloproteinases, serine protease kallikrein 6, calpain and caspases are capable of cleaving E-cadherin and thereby block the formation of stable adherens junctions[29]. The stability of the different junctional proteins is interrelated, e.g. knock-out of E-cadherin affects the formation of tight junctions and also delays the formation of desmosomes.

3.4 IMMUNE CELLS OF THE ECTOCERVIX

The local immune repertoire in the female genital tract varies depending on region (ecto- or endocervix, transformation zone or in the uterus) but there are also distinct differences between the epithelium and the underlying submucosa. $CD4^+$ and $CD8^+$ T cells scan both regions while the vascularized submucosal tissue harbors macrophages, DCs, neutrophils, natural killer cells and a few B cells, and the epithelium harbors LCs[30].

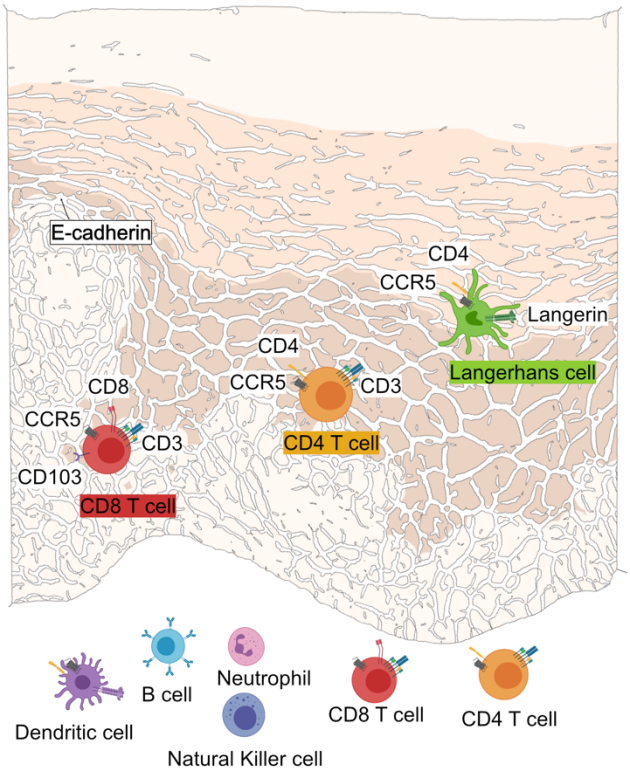

Figure 2. Illustration of the immune cells within the female genital mucosa.
The different layers of the stratified epithelium are shown in shades of beige; from the top; superficial-, upper intermediate-, lower intermediate- and parabasal layers. The E-cadherin network is shown in white. Within the epithelium there are $CD4^+$ T cells (expressing CD4, CD3, CCR5), $CD8^+$ T cells (expressing CD8, CD3, CCR5, CD103) and Langerhans cells (expressing Langerin, CD4, CCR5). In the underlying submucosa there are $CD4^+$ T cells, $CD8^+$ T cells, Dendritic cells, B cells, Neutrophils and Natural killer cells.

3.4.1 CD4+ T cells

The major HIV target cell is the $CD4^+$ $CCR5^+$ T cell. In the female genital mucosa, $CD4^+$ T-helper type 17 cells (Th17) expressing CCR6 and high levels of CCR5 and $\alpha4\beta7$, have shown to be particularly susceptible to HIV[31,32].

3.4.2 Langerhans cells

LC is a form of immature dendritic cell found in skin and mucosal epithelium and are suggested to be one the immune cells that interact with HIV in the initial step of HIV transmission[33]. HIV binding through CD4 and CCR5 molecules on LCs is suggested to lead to active infection through membrane fusion. Contrary, HIV envelope oligo-mannose binding to the C-type lectin receptor Langerin, leads to internalization and destruction in the so called Birbeck granules [33]. An infected LC easily spread the virus to the nearest lymph node and has also been shown to function as an HIV reservoir. The role of LC in HIV infection merits further attention, to elucidate if and when HIV destruction or infection is favored[34,35]. Controversially, a recent study by Pena-Cruz et al. showed that LC in the vaginal epithelium, classified by $CD1a^+$, was devoid of Birbeck granules, although contained other HIV restricting factors such as SMAHD1, that blocks the HIV reverse transcription[36].

3.4.3 Tissue resident cells

After the immune system has cleared a non-chronic infection, a long-term memory is created in both B and T cells that upon re-infection quickly can mount an immune response. Understanding this process and the phenotype of these long-term memory cells is vital in HIV vaccine development. Memory T cells can be divided into central-memory T (TCM) cells, expressing CCR7 and CD62L important for recirculation through secondary lymphoid tissues, effector memory T (TEM) cells that instead express mucosal homing markers for recirculation between blood and peripheral tissues, and a recently discovered group of non-circulating tissue resident, memory T (TRM) cells[37]. However, recent data from Fonseca et al. showed in a mouse model that virus-specific TRM cells are plastic in that they may leave the tissue site, join the circulation and give rise to TCM and TEM cells. These cells then retain a bias in repopulation of the tissue of origin upon a recall response, and in the tissue, they re-acquire their TRM signature[38].

TRM cells are found in diverse tissue sites and can be distinguished from circulating T cells based on core phenotypic and transcriptional signatures, including upregulated expression of tissue retention molecules CD69 and downregulation of sphingosine-1-phosphate receptor (S1PR1), which is required for tissue egress[39]. Moreover, $CD8^+$ TRM cells present in mucosal

and barrier sites usually express the αE(CD103)β7 integrin, which promotes tissue retention by binding E-cadherin expressed in the epithelial linings[40]. Several cytokines and chemokines, including IL-15, Transforming growth factor (TGF)-β and CXCL10, CXCL9 and CCL5, are suggested to be crucial for the migration, formation, retention, and functionality of TRM cells[41]. The anatomical localization and unique gene expression enable TRM cells to mediate local antigen-sensing and rapid protection against reinfection by promoting recruitment of adaptive and innate circulating cells to the tissue site via production of pro-inflammatory cytokines, including IFN-γ, IL-17, TNF-α and IL-2[37,42]. TRM cells may also have a regulatory function in that they express cell surface receptors known to potently inhibit T cell function, including PD-1, LAG3, CTLA-4 and CD101 and that they have the capacity to produce IL-10[37,42].

Vaccine studies in different animal models indicate that inducing a TRM response in the genital mucosa is associated with better protection against STIs including HSV-2, Simian immunodeficiency virus (SIV) and HIV[43,44] and also human papilloma virus (HPV) infection in humans[45]. Moreover, several studies are pointing towards the importance of TRM cells in the human immune response against STIs[45–48]. While Kiravu *et al* first described TRM cells in the female genital tract of HIV infected women by looking at cytobrush-derived endocervical cells and later on Moylan *et al* confirmed TRM cells in menstrual blood from HIV infected women[49,50], studies showing TRM cells in the ectocervical tissue are lacking. Better understanding of TRM cells in the female genital tract and their impact on HIV infection is key for future vaccine development.

4 THE VAGINAL MICROBIOME

The composition of the commensal microbiota that covers the cervico-vaginal epithelium impacts the health of women to a larger extent than previously thought. A *Lactobacillus*-dominant microbiome can help strengthen the barrier while a high-diverse microbiome is associated with pre-term birth, HPV- infection, persistence and progression[51,52] as well as a 4-fold risk of HIV infection[53]. Understanding how different microbial compositions affect the epithelial barrier is important to decipher what constitutes a healthy and unhealthy vaginal microbiome.

4.1 BACTERIAL VAGINOSIS

Bacterial vaginosis (BV) is one of the most common vaginal infections with a global prevalence between 23-29%[54]. The diagnosis is uncertain, about 40% of women with BV-associated bacteria display no symptoms[16]. Clinical assessment is based either on Nugents score from 1991 or the Amsel criterias from 1983. Nugents score is based on microscopic examination of vaginal smears that is graded from 0-10 dependent on presence of three bacteria morphotypes, *Lactobacillus*, *Gardnerella* and curved (*mobiluncus*-like) rods. While Amsel diagnoses BV in women presenting three out of the following four criteria; 1) Thin, white, discharge, 2) Clue cells on wet mount microscopy, 3) Vaginal pH > 4.5, 4) Release of fishy odor when adding potassium hydroxide a.k.a. the Whiff test[17]. Standard BV treatment is metronidazole that inhibits nucleic acid synthesis in anaerobic bacteria, but recurrence within one year is as high as 58%[54]. By combining sequencing-, culture-, microscopy- and epidemiological technique BV was shown to be provoked by a number of bacterial species with pro-inflammatory characteristics that depending on the host immune response gave rise to a set of common clinical signs and symptoms. Common BV-causing bacteria are *Gardnerella, Atopobium, Prevotella, Peptostreptococcus, Mobiluncus, Sneathia, Leptotrichia, Mycoplasma*, and BV-associated bacterium (*BVAB)* 1 to 3[55].

4.1.1 Bacterial vaginosis and inflammation

BV-associated bacteria are thought to origin from the gut hence they do not elicit a strong immune response. BV patients show a characteristic lack of neutrophils in vaginal fluid[56] which has been associated with both suppressed numbers of immune cells, increased CD4 to CD8 ratios, and genital inflammation[53,57,58]. Many studies show a BV-associated increase in IL-8 and IL-1β. BV has shown to increase levels of antimicrobial effectors produced by leukocytes (NO, hsp70), but also with reduction in the serine anti-protease secretory

leukocyte protease inhibitor (SLPI). SLPI is produced by both epithelial and immune cells and can block HIV entry into cells[55].

4.1.2 Bacterial vaginosis risk factors

Risk factors related to BV are smoking, low socio-economic status, douching, recent antibiotic use, and the number and frequency of sexual contacts[59]. Ethnicity may be a factor, Ravel et al. showed that American women of African descent had lower proportion of *Lactobacillus* dominance (60%) than women of European descent (90%)[60]. Similar ethnical trends have also been seen in HPV persistence and cervical cancer burden[51]. High prevalence BV regions overlap with high prevalence HIV regions, especially in sub-Saharan Africa [59]. Potential explanations for this overlap include genetic polymorphism affecting levels of TLR4, TNF-α and IL-1, predisposing women to higher-level proinflammatory responses to BV[55].

4.2 LACTOBACILLI

Lactobacilli (phylum: *Firmicutes*) dominant vaginal microbiome is associated with lower inflammation, reduced HIV-risk and a lower likelihood of genital HIV RNA shedding[61]. Of the 39 different species of *Lactobacilli* found in human vaginal samples, *L. crispatus, L. jensenii, L. iners, L. gasseri,* and *L. reuteri* are the most common[62]. *Lactobacilli* acidifies the cervicovaginal microenvironment by metabolizing glycogen-derived products to lactic acid. Lactic acid, as well as antimicrobial peptides disrupts Gram negative bacterial membrane, creating a hostile environment for many pathogenic species[17]. The shear space occupancy by *Lactobacilli* also prevents other species from colonizing the vagina. *Lactobacilli* induce regulatory T cells and are thus important in maintaining immune homeostasis in the tissue[17]. This can possibly contribute to a lower prevalence of $CD4^+$ HIV target cells in the genital mucosa and thereby to reduced HIV risk. As none of the commonly used experimental animal models have a *Lactobacillus* dominant microbiome, there is a lack of *in vivo* model systems to study the human vaginal microbiome. The *Lactobacilli* dominance in women is thought to occur during puberty upon hormone-associated epithelial changes. Increased estrogen levels cause glycogen breakdown, increase in pH as well as thickening of the vaginal epithelium, i.e. thriving conditions for *Lactobacilli*. *Lactobacilli* express pili on their surface that bind to fibronectin glycolipid receptors of epithelial cells. This is believed to favor colonization of *Lactobacilli* over other bacteria when they migrate from the rectum to the vagina[63]. Many *Lactobacillus* spp. have similar properties but there are also important inter-species differences, e.g. between *L. crispatus* and *L. iners*.

4.2.1 *Lactobacillus crispatus* vs. *Lactobacillus iners*

Lactobacillus crispatus is often found in high abundance and is considered resilient while *Lactobacillus iners* can co-habitat with a variety of species and is more often associated with a shift towards high diverse communities and BV. *L. iners* has about half the size of the genome of *L. crispatus*, and has to rely on exogenous sources of some amino acids. A unique trait of *L. iners* is the ability to produce a pore-forming cytolysin, an ability most likely horizontally transferred from *Gardnerella vaginalis*. When nutrients are scarce this may give *L. iners* a competitive advantage being able to liberate resources from host cells. This may also be the case when estrogen is low, and the microbiome is more easily disturbed. It is of interest to note that *L. iners* differentially expresses over 10% of its genome in bacterial high diverse environments (pH >4.5) including increased expression of cytolysin[64]. *L. crispatus* on the other hand has a larger metabolomic capacity and can ferment both sucrose, lactose and fructose[63]. *L. crispatus* and not *L. iners* produce hydrogen peroxide that can be harmful to other invading pathogens[17]. *L. crispatus* also produces lactocepin, a serine protease shown to degrade the proinflammatory chemokine interferon-gamma-inducible protein 10 (IP-10)[63]. Another difference between the two species is that *L. iners* can only produce L-lactic acid, while *L. crispatus* can produce both L- and D-lactic acid. L-lactic acid is associated with increased levels of MMP-8 that degrades the extracellular matrix, while a high D- to L-lactic acid ratio reduces MMP-8 levels[61].

4.3 *GARDNERELLA*

The facultative anaerobe bacteria *Gardnerella* (phylum: *Actinobacteria*) can be both gram positive and negative, and is the most virulent bacteria associated with BV. Three out of four of the Amsel criteria are based on presence of *Gardnerella*; amine odor, elevated pH and presence of clue cells[65]. Although *Gardnerella* alone is insufficient to cause BV, it is believed to be required for the occurrence of BV and is recovered from nearly all women with BV[66]. The dense biofilm that characterizes BV is dominated by *Gardnerella* in combination with other anaerobes. This biofilm is protecting the bacteria from both antibiotics and host immune responses[55]. *Gardnerella* may affect the epithelial barrier through production of cytolysin, a cholesterol-dependent pore-forming toxin, that lyses red blood cells, granulocytes and vaginal epithelial cells[65], and through production of sialidase that may be involved in degrading mucins and contribute to exfoliation of epithelial cells[55]. Zevin et al. associated *Gardnerella*-dominated microbiomes with proteomic signatures of epithelial barrier disruption even in the absence of BV symptoms[67]. As a consequence of *Gardnerella* clue cell exfoliation, studies suggest increased epithelial proliferation resulting in increased thickness

of the epithelium[68]. *Gardnerella* can be internalized by epithelial cells as shown in culture and in cervicovaginal lavage (CVL) cells of BV positive women. Internalization is thought to stimulate cytokine production but also to upregulate vimentin enabling attachment of other virulent bacteria such as *E-coli*[66].

4.4 PREVOTELLA

Gardnerella and *Prevotella* (phylum: *Bacteriodetes*) stimulate the growth of one another. Both are present in biofilm formation and in both BV symptomatic and asymptomatic women. *Prevotella* is a gram-negative anaerobe, that produces polyamines during normal metabolism leading to an increased vaginal pH. Some *Prevotella* species produce collagenase fibrinolysins and sialidase, which can damage the mucosal surface and promote the detachment of epithelial cells. *Prevotella* was shown to be the most heritable bacterial group in the vaginal microbiome and was interestingly positively linked to higher body mass index[69].

4.5 THE GUT MICROBIOME

In contrast to the vaginal microbiome, a high-diverse gut microbiome is considered healthy. Another difference is that there seem to be no genetic contribution to the gut microbiome composition in healthy women. Instead, demographic and environmental factors influence the gut microbiome, where a western diet with high proportions of fat and animal protein increase the number of *Firmicutes* (e.g. *Lactobacillus* genus) and a plant-based diet is beneficial for *Bacteriodetes* (e.g. *Prevotella* genera)[70,71]. The relationship between the gut- and the vaginal microbiome is not completely understood. The high prevalence of high-diverse vaginal microbiomes in certain geographical areas also pose the question if there is an unknown evolutionary or contextual advantage of having a high diverse microflora in the vagina, as seen in the gut.

5 HORMONAL INFLUENCES ON THE FEMALE MUCOSA

The endogenous female sex hormones estradiol and progesterone orchestrate the balance between fertilization and protection in the FGT, and have been shown to influence factors important for HIV susceptibility such as vaginal wall thickness and barrier function, vaginal pH, microbiome, cervical mucus and cervical ectopy. Several studies suggest a window of opportunity for HIV infection in the luteal phase of the menstrual cycle, when progesterone levels are high and estradiol low, coupled to a less active immune system during this phase[72]. Hormonal contraceptives contain exogenous estrogen and/or progestins that interfere with the menstrual cycle to prevent pregnancy. The progestin-only injectable contraceptive depot-medroxyprogesterone acetate (DMPA) is widely used in sub-Saharan Africa but several observational epidemiologic meta-analyses point towards an associated increase in HIV risk[73–75]. DMPA is administered every three months and progestin plasma levels are about half of peak concentration after about 20 days. While DMPA (active component = MPA) binds to the progesterone receptor (PR), it differs from other progestins and the endogenous progesterone by its additional high affinity for the glucocorticoid receptor (GR). This dual receptor affinity, and the high number of gene activation/repression effects that are induced by the binding to GR, results in a complex pattern of both immunosuppressive and immune stimulatory effects in the genital mucosa. Hapgood et al. have reviewed evidence from *in vitro*, animal and human models and concludes that DMPA modulates the structural integrity, permeability and barrier defense as well as immune factors, AMPs and the microbiota composition[76].

In humans, DMPA has only minor effects on the epithelial thickness, some studies show a decrease in epithelial glycogen concentrations causing a shift to a more high diverse microflora[23,77]. In NHP models, medroxyprogesterone acetate (MPA) is routinely used to increase infectivity of SIV by decreasing the thickness of the vaginal epithelium[78]. There are contradictory results regarding if DMPA causes an increase in HIV target cells or not. Byrne et al. showed a 3.9-fold increase in activated cervical $CD4^+CCR5^+$ T cells in the cervix and Chandra et al. presented increased numbers of vaginal CD45, CD3, CD8, HLA-DR and CCR5- expressing cells[79,80]. While Cabrera-Munoz showed decreases in CCR5 levels and increases in CXCR4 levels in PBMCs, Mitchell et al. found a decrease in $CD3^+$ and $CD3^+CCR5^+$ vaginal T cells and Smith-McCune noted increases in activated T cells in the endometrium but no increase in CCR5 expression[81–83]. HIV inhibitory factors such as SLPI and human b-defensins (HBDs) have also been shown to decrease in DMPA users[76].

6 HIV PREVENTION AND MICROBICIDES

6.1 HIV PREVENTION

In the lack of a vaccine or functional cure, different HIV prevention measures are key for containing the epidemic. For sexual transmission mechanical barriers (condoms), treatment of other STI's, reduction of local HIV target cells (male circumcision) and pre-exposure prophylactic drugs (PrEP) that prevent viral replication (containing nucleoside reverse transcriptase inhibitors (NRTI's), tenofovir and emtricitabine; sold as Truvada or Descovy) are already in use.

6.2 MICROBICIDES

Microbicides are local prophylactic compounds applied long-term or occasionally before and/or after sexual intercourse to prevent transmission of HIV and other STI. The antiviral agent can be incorporated into vaginal rings, vaginal- or rectal gels, creams or enemas. The majority of HIV prevention methods require male partner agreement to be effective, whereas microbicides offer a woman-initiated method for reducing heterosexual male-to-female transmission. A rectal microbicide would also offer a good complement PrEP method for people practicing receptive anal intercourse.

6.3 MICROBICIDES AND MUCOSAL DAMAGE

Nonoxynol-9 (N9) is a nonionic detergent, approved and used as an over-the-counter spermicide since the 1960s. N9 has also been widely used as an antiviral thanks to antiviral activities *in vitro* and in NHP studies, with remained safety according to the rabbit vaginal irritation model[84]. Paradoxically, a large clinical trial (1996-2000) enrolling 892 female sex workers showed that frequent use of N9 increased HIV risk with 50%[85]. Further studies showed that N9 increased pro-inflammatory cytokines IL-1α and IL-1β, decreased anti-inflammatory responses like SLPI[84], had a disruptive effect on the mouse vaginal epithelium (epithelium went from 40μm to 20μm)[86], and additionally caused a shift from lactobacillus-dominated vaginal microbiota to more anaerobes[87]. Another microbicide candidate, Tenofovir (TFV) 1% gel, showed 39% protective effect for vaginal use in women[88] and were found safe for rectal use in a phase II trial for MSM and transgender women (MTN-017). Despite this progress there are safety concerns related to the topical use of TFV, Hladik et al. showed that long-term TFV use suppressed anti-inflammatory mediators, increased T cell densities, caused mitochondrial dysfunction, altered regulatory pathways of cell differentiation and survival and stimulated cell proliferation in the rectal mucosa[89]. Romas et

al. associated TFV use with a mucosal protein signature related to tissue remodeling, growth and tight junction assembly[90].

6.4 MICROBICIDE SAFETY TESTING

The N9 findings raised important concerns on the safety testing of genital topical compounds. The standard of pre-clinical testing required by the US Food and Drug Administration (FDA) for spermicides and microbicides (drugs) as well as for menstrual tampons and pads (devices) is the *in vivo* rabbit vaginal irritation model (RVI) developed in 1969[91]. The RVI assess vaginal irritation by scoring immunohistochemically stained vaginal tissue for epithelial ulceration, leukocyte infiltration, oedema and vascular congestion[92]. There is a need for more precise safety evaluation methods that considers the pathogenesis of HIV. Specific guidelines for HIV microbicide development were released 2014 by the FDA proposing a number of additional safety tests that focus on cervicovaginal inflammation and epithelial breakdown[93]. There is a robust pipeline of microbicide candidates and formulations, one promising candidate being the dapivirine vaginal ring (containing a non-nucleoside reverse transcriptase inhibitor (NNRTI)) showing 30% reduction in HIV risk, high safety and ease-of-use[94,95]. Another current microbicide clinical trial initiative granted by the US National Institute of Health (NIH) is currently testing and developing new measures of microbicide safety evaluation, called the PREVENT study.

6.5 PREVENT CLINICAL STUDY

The PREvention of Viral ENTry (PREVENT) program is an integrated preclinical-clinical research and development effort to develop safe and effective rectal microbicides based on plant-produced natural viral entry inhibitors[96].

6.5.1 Lead compound Griffithsin, an HIV-binding lectin

The lead compound of PREVENT is a naturally existing protein purified from red algae (*Griffithsia* sp) called Griffithsin (GRFT)[97]. The compound was recently modified and is now assessed in the form of Q-GRFT, after introduction of an oxidation resistant modification. GRFT has shown broad antiviral effects against HIV, HPV, HSV, Nipa and MERS-CoV viruses[98–101]. Interestingly, GRFT can be produced in a tobacco plant model and thereby harvested in large quantities from tobacco plants at low cost, an important aspect for an HIV drug[102]. The HIV envelope glycoprotein evades immune recognition by hiding functionally important domains behind an oligomannose-rich “glycan shield”. GRFT binds this shield and can therefore function as a broad defense against enveloped viruses[103]. It will however be important to investigate potential cross-reactive binding of Q-GRFT to host cell surface

glycans, as such binding can lead to cross-linking of cell surface receptors and immune activation[103]. More than 24 non-clinical studies with GRFT and Q-GRFT have been performed to evaluate the tolerability and safety of the compound in accordance with the FDA guidelines. Some of these studies are presented in short below.

6.5.2 Pre-clinical testing

6.5.2.1 Cell lines and explants

First, immortalized or primary cell line cultures from tissues relevant for HIV infection were tested for viability and cytokine secretion; human cervical (Ect I/E6E7), colon (CaCo2), fibroblast (3T3) and dendritic cell lines (moDC)[103]. Next, immune activation/cytokine secretion and viability was tested in human peripheral blood mononuclear cells (PBMC) (surface activation markers CD25, CD69 and HLA-DR in $CD4^+$ PBMCs) and in cervical and colonic tissue explant, where the latter also were challenged with HIV after GRFT gel application. These data showed that GRFT had no T-cell activation, no off-target changes in gene expression, low cytokine induction and no cellular toxicity[102,103].

6.5.2.2 Rodents and rabbits

To study systemic effects of GRFT, mouse, rat and rabbit models were used, as well as mice with induced colorectal pathologies (histology, immune cell profile). These studies showed that GRFT was safe, no localized or systemic health impact, the compound was not bioavailable systemically and did not exacerbate pre-existing inflammatory pathologies. Vaginal and rectal irritation was also tested in rat and rabbit[102,104].

6.5.2.3 Non-human primates

NHPs are the only animal model for replication-competent infection of SIV and are often used for vaccine and microbicide challenge studies. Pharmacokinetic and safety assessments of GRFT were thus done in a small number of rhesus macaques (RM) (*Macaca mulatta*). GRFT concentrations after rectal applications were measured in blood samples and rectal fluids. Further exploratory studies using systems biology techniques proteomics and microbiome characterization of rectal swabs showed no safety concerns of GRFT as compared to placebo gel, although the gel itself caused minor changes[87].

6.5.3 *In situ* analysis

A relevant aspect for HIV pathogenesis is the spatial localization of HIV target cells in the genital mucosa. The RVI model counting CD45 positive cells in immunohistochemically stained tissue, and measures of secreted molecules and cytobrush-derived cells provide an

important indication on inflammatory changes in the genital mucosa. None of these techniques can however objectively and quantitatively inform on the spatial localization of specific immune cells and of the structural composition of the epithelial barrier. Digital image analysis could serve as a potential technology to answer these questions.

7 DIGITAL TISSUE IMAGE ANALYSIS

The recent boom in machine learning, computer capacity and digital scannrs has led to advances in computer vision and digital image analysis. The process of extracting data from digital images has thus become more accessible to biologist and is being applied in a wide variety of research areas.

7.1 THE PROCESS

Image analysis is the process of assigning meaning to every pixel in an image and then to classify groups of pixels into objects. Different measurements, such as shape, color and distances can then be measured on and between these objects. In tissue images, object segmentation is usually applied to delineate cells or structures. One of the main obstacles in tissue analysis is the significant variability in color intensity within and between samples. It is challenging to find rules for object segmentation that apply to a whole dataset with vast differences in staining properties. Tissue is composed of densely packed, and even overlapping cells with higher background noise and autofluorescence than cultured cells, where conditions can be streamlined. Image-based profiling of simple systems like cells, zebrafish and organoids are widely used to screen potential drug candidates for mechanism of action, target efficacy and toxicity studies[105]. Morphological changes can even be detected label-free by training a classifier to distinguish between single-cell images of cancer and normal cells for potential diagnostic applications[106]. Profiling and feature extraction in more complex systems such as tissue samples is catching up the speed, and the field of digital pathology has already an FDA approved image analysis solution for scoring of HER2 in breast cancer tissue[107]. Another internationally validated prognostic tool is the Immunoscore ®, which quantifies the density of different T cell phenotypes in the center and periphery of colorectal tumors[108]. Oncology and immune-oncology are leading the development in digital pathology followed by applications in cardiology and neurology. Digital image-based applications in infectious diseases have mainly focused on electron microscope images of viruses or live imaging of small animals, but to a lesser extent on immunopathogenesis in large sets of human samples.

7.2 HUMAN VS. COMPUTER

There is a lack of trained pathologists globally and especially in Sub-Saharan Africa and other resource-poor settings[109]. In a connected world, digital image analysis using trained classifiers makes screening and diagnosis accessible to remote areas, and when necessary, pathologist can be consulted through online solutions. Even without taking tissue biopsies, photos of intact skin and ectocervix have instead been used to reveal cellular epidermal changes or schistosomiasis infections[110].

Computerized solutions reduce human bias introduced by both visual and cognitive limitations, rendering the process objective and high-throughput. Although, human experience and ability to combine information and draw conclusions, is an advantage that needs to be transferred to the computer. It is therefore crucial for pathologists to work with computer scientists and image analysists to build the workflows and to quality control the readout parameters. For example, computers can rapidly screen large tissue samples and highlight areas that need manual investigation by a pathologist[111,112].

7.3 IMPORTANCE OF STUDYING TISSUE

Less invasive sampling of blood, cervicovaginal cells or fluids give important information on systemic or secreted immune factors, but do not necessarily mirror the state of the local tissue compartment[113]. Tissue compartments have their own immunological milieu, and studying a tissue snap-shot of that reality can give important clues for understanding the complex interplay between host factors and pathogens. For HIV pathogenesis in particular, it is important to understand the spatial localization of immune cells and potential cell-to-cell contacts, since this is a means of T cell activation and viral spread between cells.

8 METHODOLOGICAL CONSIDERATIONS

The aim of this thesis was to study the epithelial barrier, its integrity, its HIV target and effector cells, and in particular how this protective lining is affected by microbicide application (Q-GRFT, paper I), HIV infection (paper II), DMPA use (paper III) and by different vaginal microbiome communities (paper IV).

8.1 SAMPLE MATERIAL AND ETHICAL ASPECTS

For safety assessment of local microbicide products, current praxis requires animal testing prior to any clinical safety trials in humans. In paper I rectal biopsy samples from six purpose-bred RM were used. Experimental procedures in NHP models are strictly regulated to ensure the best possible treatment and care of the animals. The RMs used in our study were housed at the Center for Disease Control and Prevention (CDC, Atlanta, GA, USA) in accordance with the Guide for the Care and Use of Laboratory Animals in an AALAC-accredited facility. The animal experiments have been approved by the National Primate Research Center's Animal Care and Use Committee at the CDC in USA (CDC IACUC, protocol 2700SMIMONC).

To study TRM cells in the human ectocervix (paper II), tissue biopsies, cervicovaginal lavage as well as blood samples were collected from two separate cohorts; from Swedish women undergoing hysterectomies for non-malignant and non-inflammatory conditions at St Göran's Hospital in Stockholm, Sweden, and from HIV infected and HIV uninfected female sex workers (FSW) (Majengo Sex Worker Clinic) as well as HIV uninfected non-sex working women (Pumwani Maternity Hospital) in Nairobi, Kenya. A new study cohort was introduced four years later at the Majengo Sex Worker Clinic, Nairobi, Kenya, to study DMPA use (paper III) as well as microbiome effects (paper IV), where ectocervical tissue biopsies, CVL and blood samples were collected. All study participants were provided with written and oral information about the studies prior to confirming their participation by written informed consent. All studies were reviewed and approved by the Regional Ethical Review Board of Stockholm, the research ethics boards at Kenyatta National Hospital (Nairobi, Kenya) and University of Manitoba (Winnipeg, Canada). Biopsy sampling is an invasive method and can pose an increased risk of STIs. Therefore, detailed care was taken to explain and follow up on the agreed abstinence from sex after biopsy sampling, to ensure safety of the cervical biopsy sampling method[114]. The well-established collaboration between researchers, a strong network of peer-leaders and the study participants lay the foundation of a successful implementation of such a protocol. None of the women seroconverted to HIV after

completion of the study. Due to risk of violence, low socioeconomic status and high risk of HIV and other STI, FSW are to be considered a vulnerable population. Research including a vulnerable population is only acceptable if there is a benefit for the participants and no means of choosing another non-vulnerable cohort, according to the Declaration of Helsinki. The participants in our cohorts access free and un-stigmatized health care at the clinics and the different research programs may lead to improved HIV prevention strategies that will benefit the participants. To eliminate ethnical, geographical and other potential confounding factors in our research, it is of importance to study HIV high-risk cohorts, since they also will be prioritized users of prevention or vaccine methods. These studies would not give the same information if conducted in a non-vulnerable cohort.

8.2 IMMUNOSTAINING

Although chromogenic immunohistochemical staining gives a superior tissue morphology, immunofluorescence has less overlap between the different color spectra and is thus easier to separate and digitally analyze. All tissue biopsy material was snap frozen in optimal cutting temperature compound, sectioned in 8 μm thick sections prior to immunofluorescence staining. DAPI (Molecular Probes, Life Technologies) was used to stain the nuclei and antibodies coupled to different fluorochromes were combined for the different protein markers. Negative controls consisted of incubations in the presence of secondary antibodies alone. The stained tissue sections were scanned into digital images using a 20× objective in a Pannoramic MIDI II slide scanner (3DHistech Kft., Budapest, Hungary). The epithelial compartment was manually outlined as regions of interest in Pannoramic viewer (version 1.15.3, 3DHistech Ltd., Budapest, Hungary) on all tissue images prior to analysis.

8.3 IMAGE ANALYSIS

A central method in this thesis was to extract information from tissue images by developing image analysis workflows. In Paper I, we quantified $CD4^+$ cells and single-layered epithelial integrity in the rectal mucosa of RMs, and we developed an automated approach for compartmentalization of the rectal mucosa. In paper II we used a more specific characterization of immune cells by combining CD8 and CD103 staining and introduced a simple spatial localization method. In paper III we quantified three double stainings of potential HIV target cells, and refined the spatial localization method by compartmentalizing the stratified cervical epithelium into four layers. In paper IV we combined the refined spatial localization of CD4 cells and measures of epithelial integrity, with immune and epithelial protein markers measured in CVL samples.

8.3.1 Software

Different software offers different degrees of freedom in customization of the image analysis workflow. Commercial software, often tied to a microscope or scanner, are designed to ensure reproducibility with ready-made modules and limited customization options. We therefore chose to work with mainly open source software, CellProfiler and Ilastik, with occasional scripts in MatLab (commercial) and ImageJ. These software solutions were better suited for our discovery research approach offering high flexibility, although they require more time for training and proper understanding of the algorithms used. During the time of this thesis work, CellProfiler launched several upgrades with minor compatibility issues between each other, certain modules where therefore used in the earlier projects while the more recent projects took advantage of the most recent software versions.

8.3.2 Image preprocessing and segmentation

Tissue heterogeneity requires attention and creative solutions for image preprocessing and accurate object segmentation. Prior to starting the image analysis, the quality of the tissue, stain and scan was confirmed by trained members of the research group.

8.3.2.1 Nucleus segmentation

DAPI stained cell nuclei were segmented using three strategies. A two-step approach was used in paper I, where an image-specific threshold was used to identify pre-nuclei, separated by intensity. Each pre-nucleus was shrunk to a single pixel, and then allowed to grow as long as the input image pixels were classified as foreground (using an Otsu automated threshold), or to a maximum of 20 pixels. This two-step method gave an improved shape, that better followed the visual edge of the nuclei. For paper II a gray-level thresholding algorithm in ImageJ was applied on the raw image[115], followed by object separation using a Laplacian of Gaussian filter. In paper III and IV, nuclei were only used to define the parabasal layer (without the need to separate individual nuclei) and nuclei vs. background was segmented using an Otsu automated threshold with three classes, and the lower classes were defined as background.

8.3.2.2 Cell-based segmentation

Cell-based segmentation was used for the uniform rectal epithelial cells expressing E-cadherin in paper I, and the round-shaped $CD8^+CD103^+$ cells in paper II. Approximate outlines of cells were created using the segmented nuclei (as described above) as seeds, growing the objects until they met another object or reached a maximum allowed distance of 8 pixels. Thresholds to classify positively stained cells were visually adjusted using multiple

images from each project. Thresholds for E-cadherin were set on the mean intensity per cell, calculated on the negative isotype controls. Due to high inter-personal variation, thresholds for $CD8^+CD103^+$ cells were set based on the upper quartile intensity of all cells in the image combined with an intensity variation threshold. Positive cell counts were normalized to the total number of cells in the image or specific compartment, and were presented as cell frequency.

8.3.2.3 Pixel-based segmentation

Pixel-based segmentation has shown to be more accurate for analysis of immune cells, such as dendritic cells, with cellular protrusions reaching long distances from the nucleus, making it difficult to accurately assign pixels to a specific cell nucleus for enumeration[116,117]. Pixel-based segmentation was therefore used in paper III for CCR5, Langerin and CD3 segmentation and CD4 segmentation in paper III and IV. A white top-hat noise-reduction filter together with image-dependent intensity thresholds were used. To remove artifacts, groups of pixels < 2-5 pixels in diameter were excluded. Pixel-based segmentation was normalized to the total area analyzed, but were still presented as cell frequency.

For segmentation of CD4 cells in rectal mucosa in paper I, pixel-based segmentation was first used (as described above), followed by a cell-based cut-off where cells containing > 55 positive pixels were classified as $CD4^+$ cells.

8.3.2.4 Background issues

The rectal tissue images in paper I displayed multiple types of distinct background areas, which were identified using the pixel-based machine learning software Ilastik, and excluded from the analysis. In paper II, some samples displayed high autofluorescence in and above the basal membrane. The CellProfiler Analyst was initially assessed for training a classifier in distinguishing positive from negative cells for paper II, but when the cells were taken out of the tissue context it was difficult to categorize them without the information of the surrounding tissue. Instead, we added an additional intensity variation threshold, and thus interference from areas with uniform background intensity could be eliminated. Another way of handling diffuse background was used on the CD4 stained image in CD4-Langerin double staining (paper III). The CD4 image was first smoothed using a Gaussian Filter (size 150 pixel) and then subtracted from the original image. A common problem in paper III and IV, was the apical layers in the ectocervical tissue images that displayed high autofluorescence. This layer consists of dead cells and mucus and may unspecifically bind the antibodies. This was easily distinguished by shape/pattern and was removed manually using the EditObjects module in CellProfiler.

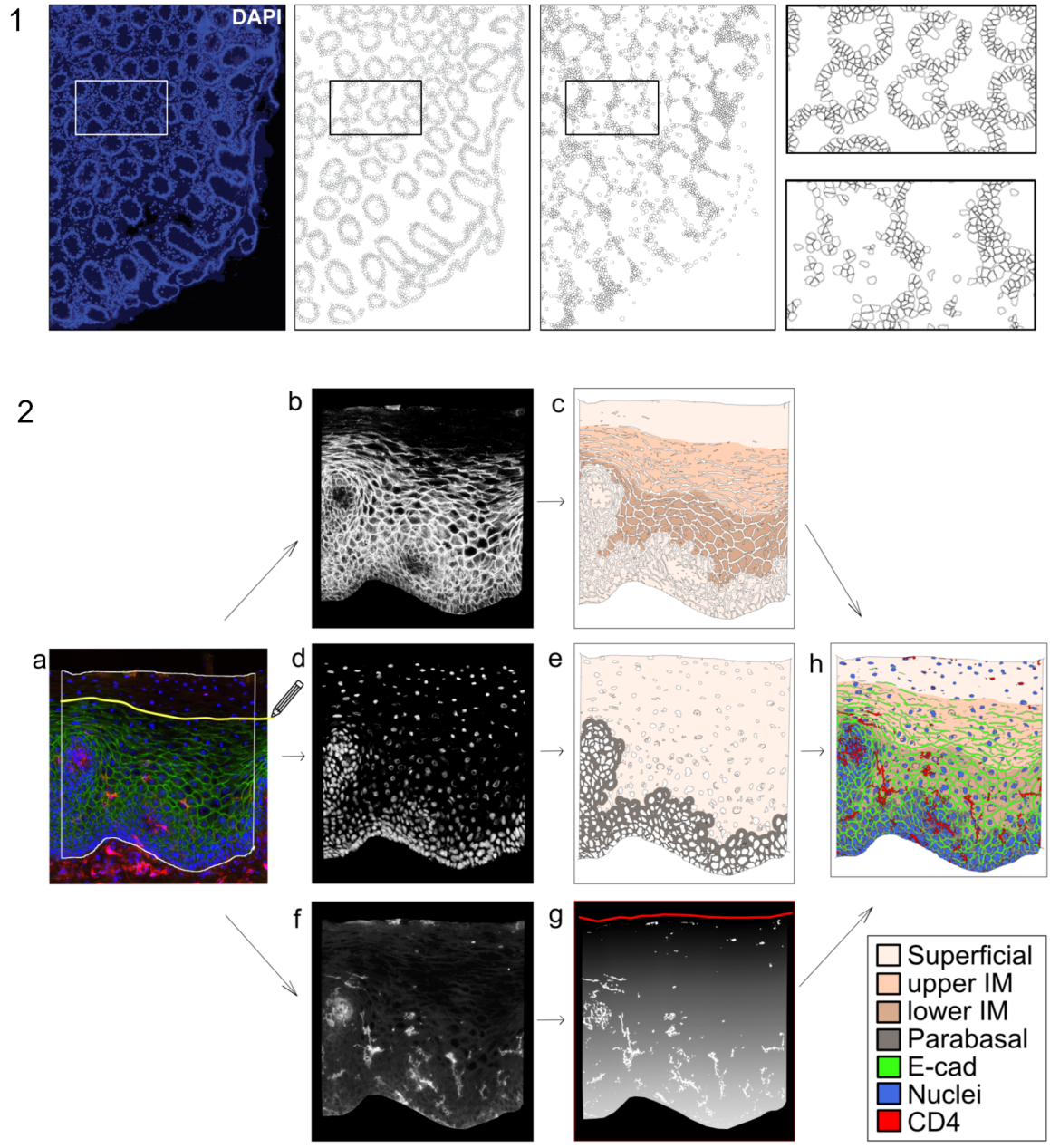

Figure 3. Compartmentalization of single layered rectal (1) and multilayered female genital mucosa (2).

(1) The DAPI stained nuclei (first column) were segmented as either epithelial cells (second column) or lamina propria (LP) cells (third column), magnified in the fourth column (epithelial cells upper image and LP cells lower image).

(2) (a) The ectocervical epithelium was manually outlined in regions of interest (white). The superficial layer was manually outlined (yellow) by following the apical border of E-cadherin staining. (b) The intermediate layer (IM) was defined using the grayscale image of E-cadherin staining, and (c) was divided into an upper "leaky" intermediate (IM) layer and a lower "intact" IM layer. (d) The grayscale image of nuclei staining was used to identify the parabasal layer, (e) which was defined by high nucleus density. (f) The grayscale image of each immune marker (here CD4) was used to quantify cell density and location in relation to the vaginal lumen. (g) The distance to the $CD4^+$ cells (white) was measured in a distance transform (illustrated by a black-to-white gradient) from the apical surface (marked in red). (h) Digital overview of the four defined epithelial layers, as well as the location of the $CD4^+$ cells and the integrity of the E-cadherin net structure.

8.3.3 Compartmentalization

One advantage with image analysis is the ability to retrieve spatial information, which can be done in different ways: For the rectal mucosa (in paper I) the epithelium was distinguished from underlying lamina propria (LP) cells by training the pixel-based machine-learning software Ilastik. Seven images, 3 positively stained and 4 negative controls were used for training. This allowed an improved output and simultaneous training of background objects. Cells were then segmented in CellProfiler and classified as either epithelial or LP, based on the probability maps exported from Ilastik (Figure 3 (1)).

For the ectocervical epithelium different methods of measuring spatial localization were explored. In paper II we measured the Euclidean distance from the basal membrane to the center of each positive cell. The research perspective in this study was to characterize TRM cells that at some point must have migrated to the epithelium from the vascularized underlying submucosa; hence a bottom up perspective was considered appropriate. This led to the discovery of different spatial niches for $CD103^{+}CD8^{+}$ and $CD103^{-}CD8^{+}$ cells in relation to the basal membrane (paper II). In paper III the pixel-based segmentation was used, therefore the distance to separate cells could not be retrieved, but instead an average distance to the parts of cells expressing the respective receptor. The research perspective in this paper was HIV risk, and since HIV enters the body through the vaginal lumen during sexual transmission, we measured the average distance to the apical surface of the epithelium. We also explored dividing the epithelium in 50µm thick objects starting from the relatively horizontal apical surface, and measuring the number of immune cells in each object (data not shown) a similar approach used by Zhang et al[118]. However due to the varied morphology of the epithelium with the sinuous-shaped basal membrane, this approach had its drawbacks. The epithelium also varies in thickness from sample to sample; a cell that is 200 µm from the apical surface can be in the middle of one epithelium, and right at the basal membrane of another. We reasoned that to capture potential biological relevant information a more refined spatial compartmentalization was needed. The epithelial cells of the multilayered ectocervical epithelium express a natural gradient of differentiation from the basal membrane to the apical dead cells facing the vaginal lumen. The epithelial junction protein E-cadherin follows this gradient, and are less expressed to become absent in the superficial layer. Since the E-cadherin staining showed a clear spatial differentiation we took advantage of this pattern to compartmentalize the epithelium into four layers, as described in paper III and IV (Figure 3 (2)). Briefly, the E-cadherin network was first enhanced using a contrast-independent approach (using MatLab) and segmented by an image-dependent Otsu

automated threshold with three classes (CellProfiler). This net-structure were then separated into two parts where the upper part (the upper intermediate layer) consisted of open net-structures, and the lower part (the lower intermediate layer) by intact net-structures, segmented by filling the holes in the net. The parabasal layer was defined based on the high nuclei density close to the basal membrane and the superficial layer was manually outlined.

All image analysis in this thesis was done on thin 2D sections of 3D samples. For distance measurements to be relevant, care was taken to cut tissue sections perpendicular to the epithelial layers. Furthermore, we assumed that the 2D view of the E-cadherin net structure provided a representative view of its local 3D structure.

8.3.4 Feature extraction and selection

For immune cells, due to tissue heterogeneity in staining intensity, we took a binary approach in classifying positive stained area or cells (cell frequency), and were not interested in measuring the scale of intensity (paper I-IV). The spatial distribution of cells was measured by calculating the percentage of total positive cells in the epithelium vs. LP (paper I) and in each of the four cervicoepithelial compartments (paper III and IV). Even though the E-cadherin staining pattern was used for compartmentalization we still evaluated the intensity and the area coverage of the net, as well as the thickness of each layer (paper III and IV).

8.4 MICROBIOME ANALYSIS

8.4.1 Nucleic acid extraction

Nucleic acid from CVL pellets was extracted using a phenol-chloroform-isopropanol (PCI) protocol[119]. Since we anticipated less bacterial DNA material from the CVL pellets compared to more frequently used vaginal swabs, the PCI protocol was chosen since it had a history in the lab of providing high DNA yields compared to commercial kits. Later research has confirmed high DNA yield with the PCI protocol from breast milk samples, although related to increased contamination[120]. Different extraction methods may also create bias for certain bacterial species. It is important to maintain strict protocols within a study to limit any technical influences. Recent research of the vaginal microbiome in NHP showed that sampling of CVL cell pellet, CVL supernatant and vaginal swabs yielded comparable profiles[121].

8.4.2 16S variable region

The bacterial 16S rRNA gene (approx. 1500 basepair long) contains nine hypervariable regions (V1-V9) with interspersed conserved regions that can be used for universal primer sequences across species. Illumna MiSeq offers read lengths up to 300 basepair and is often

used for sequencing of specific regions which reduces the cost. Although, for accurate species taxonomy of all bacteria, all nine variable regions are needed. For example, V1-V2 underrepresent *Gardnerella* but offer higher species resolution for *Lactobacillus* spp. V3-V4 are suggested to best capture the vaginal microbiome repertoire[122]. Here we used V4 region (515F/806R primer set) for practical reasons.

8.4.3 Cervicotyping

There is so far no consensus in the grouping of vaginal microbiome clusters. Depending on the population and the research question, different cervicotype (CT) groups (also called vaginal microbiome groups, community state types) are being used. The CT classification described by Gosmann et al, showed a significant difference in HIV acquisition between groups[53], therefore this classification was used to assign our study subjects to one of the four CT groups based on their cervicovaginal microbiome composition.

8.5 PROTEIN PROFILING

To complement the image analysis data, we assessed protein markers of epithelial integrity and inflammation, in CVL samples. We selected a targeted protein approach detecting 63 proteins previously described in genital fluids, with associations to HIV resistance and inflammation[123]. The assay was performed at a core facility SciLifeLab, using a high-throughput bead-based affinity assay, based on Human Protein Atlas antibodies and a FlexMap 3D instrument (Luminex Corp., Austin, Unites States). Antibody-based assays are dependent on the quality and availability of antibodies, and targeted approaches are by default limited to selected markers. Although, the targeted panel used here has been carefully selected based on mass-spectrometry data and was thus considered both time-efficient and less costly compared to a discovery approach, and was well-suited to our needs.

8.6 STATISTICAL METHODS

To minimize the number of RMs used in paper I, the clinical trial design was set up to re-use the individual RMs for both baseline, placebo and Q-GRFT treatment assessments. First, baseline levels were assessed by collecting 2x7 samples from each of the six NHPs over nine weeks. After two weeks of resting the same RMs were given placebo treatment at two timepoints (2x2 samples) followed by six sampling time points after Q-GRFT treatment (2x6 samples) spread out over 16 weeks. The genetic and environmental differences between the RMs were kept to a minimum, although this set up required accounting for potential confounders of resting time between sampling, and the accumulating number of biopsy samplings throughout the study. A linear mixed effects (lme) model was used to account for the confounders. Incorporating many fixed effects in a lme model on a small sample

population (n=6) involves a risk of diluting their contribution on the outcome variable. Adding and removing effects from the model gave us confidence in the final model and we deemed it necessary to use a lme model to account for the trial design in our analysis.

Comprehensive clinical and socio-demographic parameters were collected from all human study participants in paper II, III and IV. Relevant parameters were compared between > 2 groups by two-sided Kruskal–Wallis Rank Sum test (paper II and IV), and for 2 groups by Mann-Whitney U test (paper III) for continuous data and Fishers' chi-squared test for categorical data (paper II, III and IV). In paper II, there were two HIV seronegative control groups that were compared against the HIV infected group, a low-risk non-sex working group and a sex-working group. Comparisons between the infected and uninfected FSW group were assumed to account for potential effects caused by sex work per se, thus no multivariate analysis was performed. There were no significant differences in clinical parameters between groups in paper III and IV, therefore no multivariate analysis was performed. For analysis of 63 proteins in CVL samples, Kruskal-Wallis calculated p-values were adjusted for multiple comparisons using Benjamini-Hochberg correction. Multiple comparison corrections were not used for the 55 measurements of protein frequency, spatial localization, proportions, assessed by image analysis in paper III, while this was later implemented for 16 similar measurements in paper IV. Spearman's rank correlation coefficient test was used to assess correlations in paper II, III and IV.

9 RESULTS AND DISCUSSION

9.1 PAPER I

The use of new technology has revealed the complexity of the human immune system. This gives us the possibility to develop more refined vaccines and other interventions. Previous safety issues with N9 and findings regarding the microbiome effect on TFV (CAPRISA004) has caused the FDA to broaden the repertoire of safety assessment methods for new microbicide drugs[93,124]. Especially methods focusing on inflammation and epithelial breakdown. Our aims in paper I were to evaluate image analysis as an *in situ* assay for safety assessment, and to assess potential mucosal changes in NHP after rectal administration of the Q-GRFT microbicide.

An image analysis workflow for compartmentalization of rectal mucosal cells as either epithelial or LP cells was created. $CD4^+$ and E-cadherin$^+$ cells were quantified in each compartment. Rectal biopsies display a high level of variation in their morphology, depending on how the tissue villi are oriented at biopsy sampling, fixation and tissue sectioning. By normalizing the data within each tissue compartment, i.e. against the number of epithelial and LP cells, and not just the total number of cells or tissue area analyzed (which is common), a higher precision was achieved. Such precision and spatial information is lost when applying flow cytometry or transcriptomics analysis on digested tissues, or when measuring immune cells in cytobrush-derived samples. The automated image analysis developed here offered comparable throughput as flow cytometry. A total of 8.7 million cells covering a surface of 10.8 cm^2 in the 180 tissue images analyzed were objectively analyzed (6 RMs x 2 biopsies x 15 timepoints).

As a proxy for epithelial integrity, the number of E-cadherin$^+$ cells out of total epithelial cells was quantified. Three different intensity thresholds to define an E-cadherin$^+$ cell were evaluated, all of which showed stable E-cadherin expression across the experiment (data not shown). The frequency of E-cadherin$^+$ cells in the epithelium remained constant despite application of placebo or Q-GRFT gel, and was neither affected by multiple biopsy sampling interventions.

Tissue compartmentalization enabled separate enumeration of $CD4^+$ cells within the epithelial compartment and within cells residing in the LP. This is biologically relevant information since intra-epithelial $CD4^+$ cells are in closer proximity to incoming virions and

may thus pose a larger HIV risk compared to cells in the underlying LP. Multi-dose application of Q-GRFT (four days in a row) caused a small, but significant increase of the frequencies of both intra-epithelial $CD4^+$ cells (placebo: median 4%; Q-GRFT (1%): median 7%) and $CD4^+$ cells residing in the LP (placebo: median 30%; Q-GRFT (0.1–1%): median 36–39%). As a proxy for inflammation the total number of cells in each compartment was quantified and did not change after Q-GRFT treatment. This indicates that the increase of $CD4^+$ cells may represent upregulation of the CD4 marker rather than an influx of $CD4^+$ cell. Some indications of inflammatory responses were seen in the pre-clinical tests, e.g. the GRFT-P (0.1%) had twice as high RVI score compared to sham treated rabbits (score 2 vs. score 1), although these scores are well within the limits for clinical testing of vaginal products (RVI < 8)[102]. Another study showed that the gel formulations in themselves (HEC and Carbopol) were associated with temporary alterations in expression of proteins involved in proteolysis, and in activation of the immune response and inflammation[87]. The sensitivity of our image analysis workflow may thus have picked up subtle changes that the safety assessment in other model systems such as the RVI model did not see. Although, the biologic relevance of the minor increase of $CD4^+$ cells observed in the rectal mucosa with multiple Q-GRFT dosing is likely to be negligible.

Using a linear mixed effects model allowed us to investigate changes in epithelial and HIV target cell markers while considering potential effects of multiple sampling (2 biopsy/timepoint x15), resting time (after previous biopsy sampling time point) as well as the placebo or Q-GRFT treatment including interindividual variation. Remarkably, samples taken from untreated RMs after a resting period of 14 days compared to 7 days showed a reduced frequency of $CD4^+$ cells, both in the epithelium and in the LP, as well as a decrease in the total number of LP cells. There was also a decreasing trend in $CD4^+$ cells over time in untreated RM, suggesting that the mucosa showed a possible habituation to the sampling procedure and reduced response, or a reflection of strong reaction to the initial rectal fluid and swabs that were taken frequently during the first 24 hours. Interference of sampling itself is difficult to study. Dezzutti et al. noticed a small change in viral replication in vaginal and cervical biopsies taken from women who had been sampled before compared to women who were sample for the first time[125]. Patton et al. investigated epithelial shedding in rectal lavage samples and noticed that uncareful insertion of the collection syringe itself caused epithelial shedding, which later was avoided by a more careful sampling procedure[126].

A caveat in our study design is the longitudinal sampling and treatment of the same RM as well as the non-homogeneous resting time throughout the study. Despite the use of a linear mixed effects model, assessing a limited number of subjects (n=6) with multiple fixed effects makes it challenging to interpret the true impact of each separate effect. To further understand the biological relevance of the changes in $CD4^+$ cell numbers, it would be important to evaluate a wider range of drug (Q-GRFT) concentrations, a mechanical control (mock gel), and a positive control known to increase HIV or SIV acquisition *in vivo*, such as imiquimod or N9[84,85]. Further improvements such as adding distance measures, to quantify cell-cell proximity or distance from immune cell to lumen, and/or increasing the number of stained markers, would allow a more detailed phenotypic analysis and create a map of the immune cell landscape in the mucosa. Especially of interest would be to see if the $CD4^+$ cells represent the activated $CD4^+CCR5^+CCR6^+$ Th17 phenotype which has been shown to correlate to SIV acquisition in an experimental vaccine model in RMs[127]. Although, there are no CCR5 antibodies available for NHP tissue.

In summary, the frequencies of rectal E-cadherin$^+$ cells remained stable despite multiple tissue samplings and local application of Q-GRFT, whereas minor increases in the frequencies of rectal mucosal $CD4^+$ cells occurred after multiple Q-GRFT applications. The resting time between sampling points were further associated with minor changes in the total and $CD4^+$ rectal mucosal cell levels and needs to be observed in future studies. Although statistically significant, the biological relevance of the minor changes in $CD4^+$ cell frequency for mucosal HIV susceptibility is most likely negligible, but needs attention in a future clinical trial. Image analysis of mucosal tissues thus offers high-resolution, quantifiable, spatial information on cellular and structural changes that cannot be achieved with any other technology. The method can theoretically be applied on a variety of samples throughout the clinical trial process, tissue explants, rodent/rabbit, NHP and human samples. Although, biopsy sampling in large clinical trials in a human HIV high-risk cohort would pose an increased infection risk. The image analysis platform described here therefore offers a versatile complement to traditional methods of safety evaluations, well suited for pre-clinical and smaller phase I human trials, by highlighting cellular markers of relevance for HIV transmission.

9.2 PAPER II

Tissue resident immune cells with an effector memory phenotype have a rapid response against re-infections and may be important against HIV[128]. The aim of paper II was to investigate if $CD8^+$ T cells residing in the ectocervical epithelium displayed a tissue-residing phenotype, and if HIV infected women had an altered phenotype of these cells.

While two previous studies describe TRM cells in the female genital tract of HIV infected women, these studies are based on cytobrush-derived endocervical cells and menstrual blood[49,50]. We here presented the first study that showed $CD8^+$ TRM cells in cervical tissue biopsy samples from HIV infected women. $CD8^+$ TRM cells were first characterized in ectocervical tissue samples from healthy Swedish women using flow cytometry. The majority of $CD8^+$ T cells in the epithelium (85%) were effector cells ($CD45RA^-CD62L^-$), and 84% and 76% expressed the TRM markers CD103 and CD69, respectively. The expression of CD103 was significantly higher on $CD103^+$ $CD8^+$ T cells in the epithelium compared to both submucosa and blood, as could be expected since CD103 binds to E-cadherin present in the epithelium.

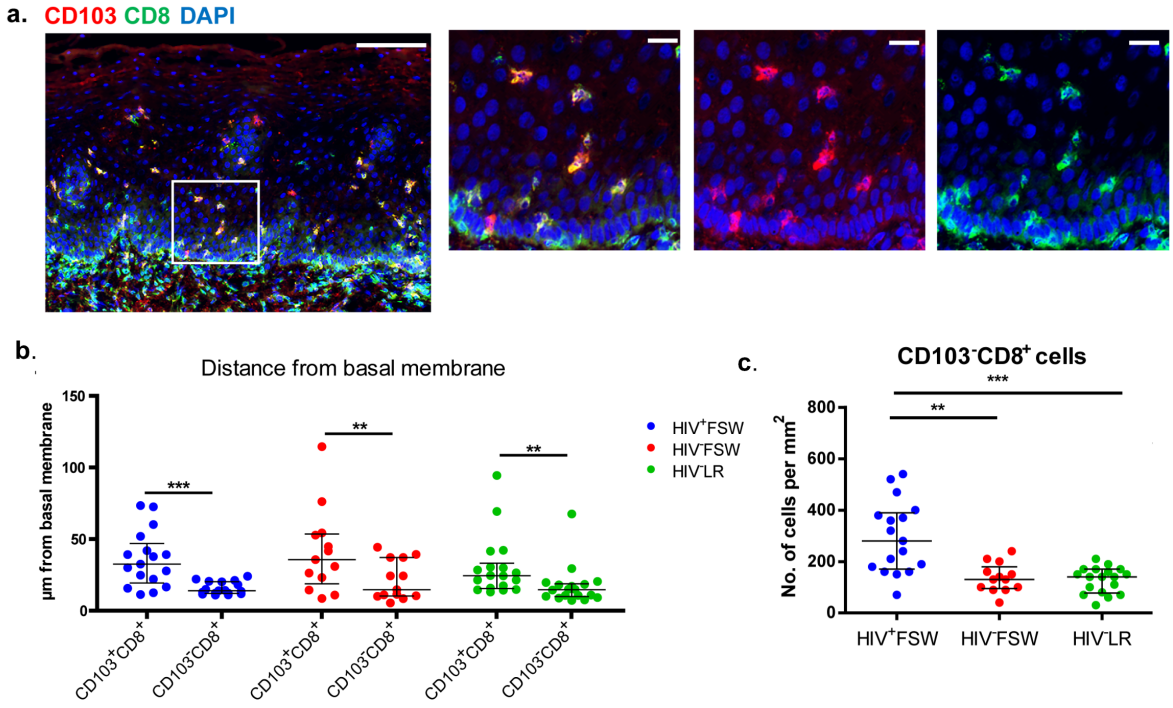

Figure 4. Visualization and enumeration of $CD103^{+/-}CD8^+$ cells in the ectocervical epithelium.

*(a) Representative immunofluorescence images from an HIV^+FSW, stained for $CD103^+$ (red) and $CD8^+$ (green); double-positive cells appear as yellow, nuclei (blue). Magnifications of the region in the box in the image to the left (scale bar 100 µm) are shown to the right (scale bar 20 µm) (b) Scatter plots showing the distribution of the median distance of cells to the basal membrane of the ectocervical epithelium. c) Scatter plots showing numbers of $CD103^-CD8^+$ cells/mm2 tissue area. Horizontal lines represent median ± interquartile range. Statistical significance was determined using the Kruskal-Wallis test, followed by Dunn's post hoc test; **, $P < 0.01$ and ***, $P < 0.001$.*

The profiles of TRM cells during a chronic infection was investigated by comparing HIV infected women and un-infected controls, and showed that HIV infected women had elevated frequencies of $CD103^{-}CD8^{+}$cells (Figure 4c). Applying digital image analysis to immunostained tissue sections revealed that the $CD103^{-}CD8^{+}$cells resided closer to the basal membrane of the ectocervical epithelium, compared to the more apically located $CD103^{+}CD8^{+}$cells (Figure 4b). This suggests a recent influx of these cells over local proliferation, and is supported by immunostaining showing that neither $CD103^{+}$ nor $CD8^{+}$ cells co-expressed Ki67. HIV infected women have an activated immune system both systemically and locally[129,130], and our data showed that HIV infected women had higher mRNA levels of TGF-beta, CXCL10, CXCL9, CCL5; chemokines important for tissue migration of T cells. High levels of some of these chemokines correlated with high levels of $CD103^{-}CD8^{+}$cells in the cervical epithelium as well as with plasma and cervicovaginal secretion viral load. We speculate that the enhanced immune activation and viral replication is a driving force for $CD103^{-}CD8^{+}$cell recruitment. Another study by Kiniry et al. also showed low CD103 expression on $CD8^{+}$ TRM cells in rectosigmoidal mucosa of treatment-naïve HIV infected men, and these cells furthermore accounted for the majority of HIV-specific cells, suggesting an HIV-associated alteration of the TRM subset[131]. In line with our findings, it has also been shown that chronic antigenic stimulation reduces CD103 upregulation on antigen-specific $CD8^{+}$ T cells in mice[132]. Antigen re-activated $CD8^{+}$ TRM cells are important for the recruitment of systemic memory cells to the site of infection[131]. We showed that HIV infected women had reduced CD103 expression on circulating $CD8^{+}$ T cells in both the transitional and effector memory pool compared to controls. They also displayed enriched $CD103^{+}CD8^{+}$cells within the effector memory pool, presumably due to the high antigenic burden in HIV infected women. These cells were highly activated and displayed elevated levels of PD-1 exhaustion marker.

This study is to our knowledge also the first to show and digitally quantify the spatial localization of immune cells by immunostaining in ectocervical tissue. One previous study has investigated tumor-infiltrating TRM cells in cervical cancer samples (n=460) by compartmentalizing tumor and surrounding tissue and manually counting cells from five selected immunostained tumors[133]. In our samples the morphology of $CD8^{+}$ T cells allowed digital identification of positive cells, by measuring stained intensity in an area slightly larger than each nucleus. Challenges with this method was to handle positive cells that were in the junction of several nuclei, where general segmentation rules categorized all cells as positive. We used two thresholds to reduce unspecific background, a general image dependent

intensity threshold as well as an intensity variation threshold that removed evenly distributed background across cells. Despite these thresholds, there was a lot of variation across samples resulting in visually assigning samples to either a higher or a lower set of thresholds. To confirm that this manual step did not infer any bias, all samples were run using both the higher and the lower thresholds resulting in the same significant differences between the groups. This supports assigning one threshold to a large number of images despite minor variation in precision. Finding a single threshold that worked for all tissue samples was challenging, but on a group level we could still see a difference regardless of threshold used. The drawback is the risk of averaging out potential differences. For correlation of individual TRM counts to cytokine levels we used the mix of higher and lower thresholds for the tissue samples since our study material can be considered small (n=17). For larger studies this should not be necessary. The clinical parameters for the women in the cohort showed that HSV positivity and vaginal douching were significantly different between the low-risk non-sex working group and the two sex-working groups. Even though comparisons between the two FSW groups showed similar results as between the HIV infected group and the non-sex working group, it would have been interesting to account for these confounders by setting up a multivariate analysis.

In summary; we here showed that $CD8^+$T cells in the ectocervical mucosa present tissue residing markers CD103 and CD69. HIV infected women showed an altered phenotype of these cells compared to uninfected controls; within the circulating effector memory pool they showed higher levels of $CD103^+CD8^+$cells, and reduced levels of $CD103^-CD8^+$cells, both in circulation and within the epithelium. In the epithelium the $CD103^-CD8^+$cells resided closer to the basal membrane suggesting a recent influx of these cells in HIV infected women.

9.3 PAPER III

The efficient and easy-to-use hormonal contraception DMPA is widely used, especially in HIV endemic areas[134]. This poses a potential public health problem since studies indicate a correlation between DMPA use and increased HIV risk[74,75,135]. It is therefore important to determine the effects of DMPA use at the cellular and molecular level in the female genital mucosa. Previous studies have focused foremost on protein analyses in genital secretions and cellular analyses of cytobrush-derived cervical mononuclear cells[80,82,83]. We here aimed to add quantification of various subtypes of HIV target cells and their spatial localization in cervical tissue as well as a thorough characterization of the epithelial barrier.

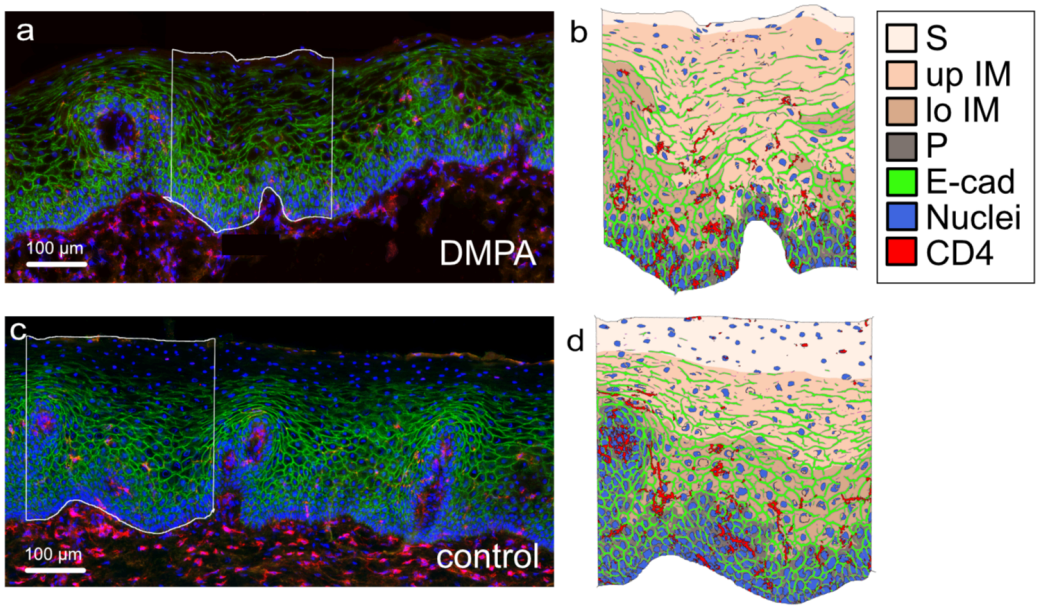

Figure 5. (a) Representative image of immunostaining from a woman using DMPA, (b) enlarged digital image showing the four epithelial layers including the thin superficial layer and the presence of $CD4^+$ cells in the upper intermediate layer. (c) Representative image from a woman not using a hormonal contraceptive (control), and (d) enlarged digital image showing the thicker superficial layer and a more basal localization of $CD4^+$ cells. S = superficial, up IM = upper intermediate, lo IM = lower intermediate, P = parabasal layer.

In this study we therefore further developed the concept of tissue compartmentalization and spatial localization of immune cells. Epithelial cells in the stratified ectocervical epithelium are in a state of transition, resulting in layers of phenotypically different cells. To account for this continuous cell renewal as well as the varying morphology of the epithelium, we defined four epithelial layers. We took advantage of the high nuclear density in the cell layers close to the basement membrane to define the parabasal layer. Furthermore, we defined the lower intermediate layer (IM) by their intact E-cadherin expression, the upper IM layer by cells with down-regulated or broken E-cadherin junctions, and the superficial layer which lacked E-cadherin expression. The varying differentiation of the cells in the epithelium entails horizontal sub-niches with specific characteristics. For example, keratin 19 stains basal cells,

keratins 5 and 14 are expressed in parabasal cells and weakly in intermediate cells and rarely in superficially cell layers[24]. Murall et al. emphasize the importance of considering epithelial stratification in understanding disease patterns. Their mathematical model recreates epithelial and infection dynamics when examining infection differences in high- or low virulent HPV, that cause basal-up infections compared to *Chlamydia* bacterial infections that spread surface-down and are lytic[136]. They suggest that the duration of infection can be driven by the rate at which the stem cells of the epithelium divide. Anderson et al. also highlights the biological differences of the different epithelial compartments, zooming in on the stratum corneum/superficial layer that retains innate and adaptive functions creating a specific niche[23] with implications for pathogen defense.

A thicker stratified epithelium is likely to offer a better protection against entry of pathogens. Estradiol has a known thickening effect of the epithelium related to an increased storage of glycogen within epithelial cells. Menopausal women, with low estradiol levels, have a thin and more brittle cervicovaginal epithelium with higher prevalence of BV and other complications[137]. DMPA use has not shown significant reduction of epithelial thickness in most human studies[79,138] but has a strong effect on the NHP genital mucosa where MPA is used to promote SIV infection[78]. In concordance with previous studies the total epithelial thickness in our cohort was comparable between DMPA users and women not using any hormonal contraception (the control group). Nonetheless, our detailed epithelial compartmentalization revealed that the superficial layer was significantly thinner with an average of 50µm in the DMPA group compared with the control group (illustrated in Figure 5). A similar finding has been shown in women using progestin intra-uterine devices[139]. A potential increased risk of DMPA users may be the lack of this superficial layer niche. Free viruses are thought to diffuse on average 10µm into this layer and maximum up to 50µm, which correspond well to the reduced thickness of DMPA users. Some of the women displayed a complete lack of the superficial layer which would indicate a direct access for viruses to viable epithelial cells. These cells are capable of mounting an immune response which in turn leads to potential influx of HIV target cells.

DMPA did not affect the integrity of the epithelial barrier since both groups displayed an equally intact E-cadherin junction network. Interestingly, we found that systemic estradiol levels measured in the control group were positively correlated to E-cadherin expression in all three epithelial layers indicating a stronger epithelial barrier in women with high estradiol levels. Estradiol levels were also negatively correlated to the height of the parabasal layer.

Endogenous progesterone levels on the other hand did not correlate to any measures of thickness and integrity of the epithelium. Among several effects, hormones can influence E-cadherin through physiological mechanisms, such as progestin-mediated transcriptional regulation[140], and through changes in the microflora and mucosal protein expression[77,141,142].

We hypothesize that HIV target cells located closer to the basal membrane, i.e. further away from the vaginal lumen in the parabasal area with strong adherens junctions, are harder to reach for an incoming virus, as long as the epithelium remains intact. Our data showed that both DMPA use and systemic levels of estrogen, but not endogenous progesterone, had an impact on the spatial localization of HIV target cells. DMPA use was associated with a higher proportion of $CD4^+CCR5^+$, $CD4^+Langerin^+$ and $CD4^+CD3^+$ cells in the upper IM layer where adherens junctions are less intact, compared to controls. A plausible mechanism could be the reduced superficial layer in DMPA users leading to an inflammatory response that induces movement of HIV target cells upwards to the superficial layers. Correlating superficial layer thickness to cytokine and chemokine levels would be of interest, although these measures were not included in this study. Previous studies have shown both increases in pro- and anti-inflammatory mediators in DMPA users[76,140,143]. It is not clear if these effects seen by DMPA use is caused by the progestin compound or the hypoestrogenic state it induces. By correlating systemic hormone levels measured in the control group to HIV target cell localization, we showed that estradiol levels were positively correlated to a more basal location of both $CD4^+CCR5^+$ and $CD4^+Langerin^+$ cells, i.e. a shift from the lower IM to the parabasal layer. There were no correlations to systemic progesterone levels. These data imply that it may be the hypoestrogenic state in DMPA users that lead to more accessible HIV target cells.

The high affinity of the DMPA-progestin for the GR and PR infer both trans-repression and partial trans-activation of hundreds of genes related to immune cell function, trafficking and proliferation[76]. This complex hormonal activation and regulation makes it difficult to decipher a simple mode-of-action for DMPA. Many but not all studies have associated DMPA-use with increases in HIV target cells in the female genital tract, such as activated $CD4^+$ cells and macrophages[79,81,83]. By quantification of cells in the epithelium we here showed that the frequency of $CD4^+CCR5^+$ as well as $CD4^+CD3^+$ cell out of the total $CD4^+$ cell pool was increased in DMPA users compared to controls. Furthermore, the DMPA group showed a lower frequency of $CD4^+Langerin^+$ cells, in contrast to previous studies that showed a hormone related increase in the frequency of vaginal LCs in low estrogen, high

progesterone conditions, as well as a DMPA study where no difference in vaginal LCs between DMPA users and controls were seen[76]. The pixel-based quantification from our *in situ* image analysis showed a median value of 38% for DMPA users and 24% for controls, of total CD4 cells co-expressing CCR5. The numbers we obtained with image analysis are in the ballpark of two previous studies using flow cytometry on ectocervical tissue (epithelium and submucosa) from hysterectomy patients showing 50%[144] and 28%[30](in both pre- and postmenopausal women) of CD4 T cells expressing CCR5, respectively.

Some potential limitations working with human tissue samples include inter-subject variation in staining intensity and the natural variability of the tissue morphology. Duplicate samples would have allowed a more robust estimation of measurement from each woman. However, since we are working on tissue material from a HIV high-risk cohort, this was not feasible. For ethical and clinical reasons, we were limited to one biopsy per study subject for image analysis. An ideal, but not feasible, study design would have included tissue material to also enable analysis by flow cytometry. This would have allowed a more precise assessment of cell subsets using multiple markers on individual cells. In total we analyzed 55 measurements of protein frequency, spatial localization, proportions, assessed by image analysis. We did not correct for multiple comparisons since we assessed this to be a targeted approach and also since it is debatable at what number of measurements a multiple comparison correction is needed. For the sociodemographic and clinical parameters collected, there were no differences between DMPA users and controls. Although, it could be of interest to set up a multivariate model accounting for these parameters, which may reveal certain parameters' (e.g. age and time in sex work) impact on our measured variables. This could potentially reveal new patterns and significant differences between the groups. A disclaimer with clinical and socio-demographic data, is that despite thorough questionnaires there is always potential confounders that are not included, and self-reported data can also contain bias or self-censored information.

In summary, we here developed a method to study sub-compartmentalization of the ectocervical epithelium by using digital image analysis. This method revealed that DMPA users had a higher proportion of HIV target cells located closer to the vaginal lumen, a higher frequency of $CD4^+$ T cells ($CD4^+CD3^+$) and $CCR5^+CD4^+$ cells out of the total $CD4^+$ cell pool in combination with a thinner superficial layer. All these observations indicate a potential mechanism behind an increased HIV risk for DMPA users.

9.4 PAPER IV

Women with a high diverse vaginal microbiome have four times higher risk of acquiring HIV as well as increased risk of other adverse health outcomes such as pre-term birth and HPV infection, compared to women with a lactobacillus dominant microbiota[51–53]. Signs of inflammation and epithelial damage have been noticed in DMPA users by characterizing protein profiles of vaginal secretions[140]. In paper IV we aimed to investigate the effects of the microbiome composition on epithelial markers of integrity and HIV target cells *in situ*. Furthermore, a selected protein panel was used for correlation of *in situ* patterns to secreted markers of epithelial disruption and inflammation.

Microbiome composition was measured in DNA extracted from CVL cell pellets, by sequencing of the 16S variable region 4. The women were divided into four cervicotype groups (CT) based on their microbiome composition; *Lactobacillus non-iners* dominant, i.e. mainly *L. crispatus* dominated, (CT1), *Lactobacillus iners* dominant (CT2), *Gardnerella* dominant (CT3) and high diverse (CT4). The female sex workers in our cohort showed similar distribution pattern of their cervicovaginal microbiome as previously reported for women of African descent. A total of 45% of the women in our cohort had a *Lactobacillus* dominant microbiome (Figure 6b), in parity with 40% of African-American women[60], 50% of Dutch women of Ghanaian descent[57], 37-45% of South African women[145,146] and 46% of Rwandan female sex workers[61]. These comparisons do not single out sex workers as having a higher diversity, in contrast to a study by Wessels et al. which in a similar cohort of Kenyan female sex workers showed that only 17% were *Lactobacillus* dominant as compared to 58% in a control group of non-sex-workers[147].

Applying our epithelial sub-compartmentalization method developed in paper III we characterized the epithelial integrity and compared the four CT groups. Our data showed that *Lactobacillus non-iners* dominant women (CT1) displayed a more robust epithelial barrier as defined by E-cadherin expression compared to the other three community types. The upper IM layer consists of cells with down-regulated or broken E-cadherin junctions, these cells will further downregulate these junctional proteins when they differentiate and move up into the superficial cell layers. The CT1-group displayed a thinner upper IM layer, i.e. had fewer cell layers with broken E-cadherin compared to both *L. iners-, Gardnerella-* and high diverse-communities (CT2-CT4). The E-cadherin junctions remaining in these "broken" cell layers also covered a larger area in women with *L. non-iners* dominant (CT1) microbiome, supporting a more stable epithelium in this group. This may reflect that bacteria in CT2-CT4

groups cause a direct or indirect down-regulation of the E-cadherin junction proteins or totally degrade them. In line with our findings, another study showed that women with high diverse microbiome had increased levels of cytoskeleton rearrangements and actin-organizing proteins, decreased keratins and cornified envelope proteins, reflecting epithelial damage and/or remodeling[16]. The same study showed that increasing bacterial diversity led to increases in cell death as measured by lactacte dehydrogenase subunits A and B. These functional characteristics of the genital mucosa from bacterial high diverse women were also addressed in studies by Yeoman et al., who showed that women with BV had altered cell-wall associated proteins suggesting loss of epithelial integrity[148], and by Zevin et al. who showed that *L. iners* and *G. vaginalis* dominated women had an increase in enhanced membrane transport and secretion of extracellular products compared to *L. crispatus* group[67]. These data collectively conclude that a high diverse microbiome affects the epithelial protein composition.

Even though *Lactobacilli* is considered beneficial, there is accumulating evidence that different *Lactobacilli* species exhibit distinct properties. For example, *L. crispatus* dominated cervicotype, but not *L. iners* dominated group was associated with lower HIV acquisition rates as compared to the high diverse group[53], for reasons not yet identified. Interestingly, our image data showed that *L. non-iners* had significantly fewer cell layers with broken E-cadherin junctions than the *L. iners* group. Potential reasons for this discrepancy could be due to the fact that *L. iners* but not *L. crispatus*, are capable of producing a pore-forming cytolysin that besides disrupting the cells may have effect on their E-cadherin expression. *L. crispatus* produce D-lactic acid which prevents the EMMPRIN induced MMP-8 to breakdown the extracellular matrix, whereas *L. iners* lack this ability and only produces L-lactic acid which instead is positively correlated with EMMPRIN levels in vaginal fluids[149]. Our data also showed a non-significant trend that the high-diverse CT4 group had fewer cell layers with broken E-cadherin compared to *L.iners* (CT2) and *Gardnerella* (CT3) groups, supporting the cytolysin theory, since the cytolysin producers *L. iners* and *Gardnerella* are minor parts of the community in the high diverse CT4 group.

In terms of difference in the secreted protein profile between the two *Lactobacilli* groups, there were only two significant proteins. *L. iners* group had significantly higher CVL levels of the epithelial barrier protein SPRR3 and of the protease inhibitor ITIH2 compared to *L.*

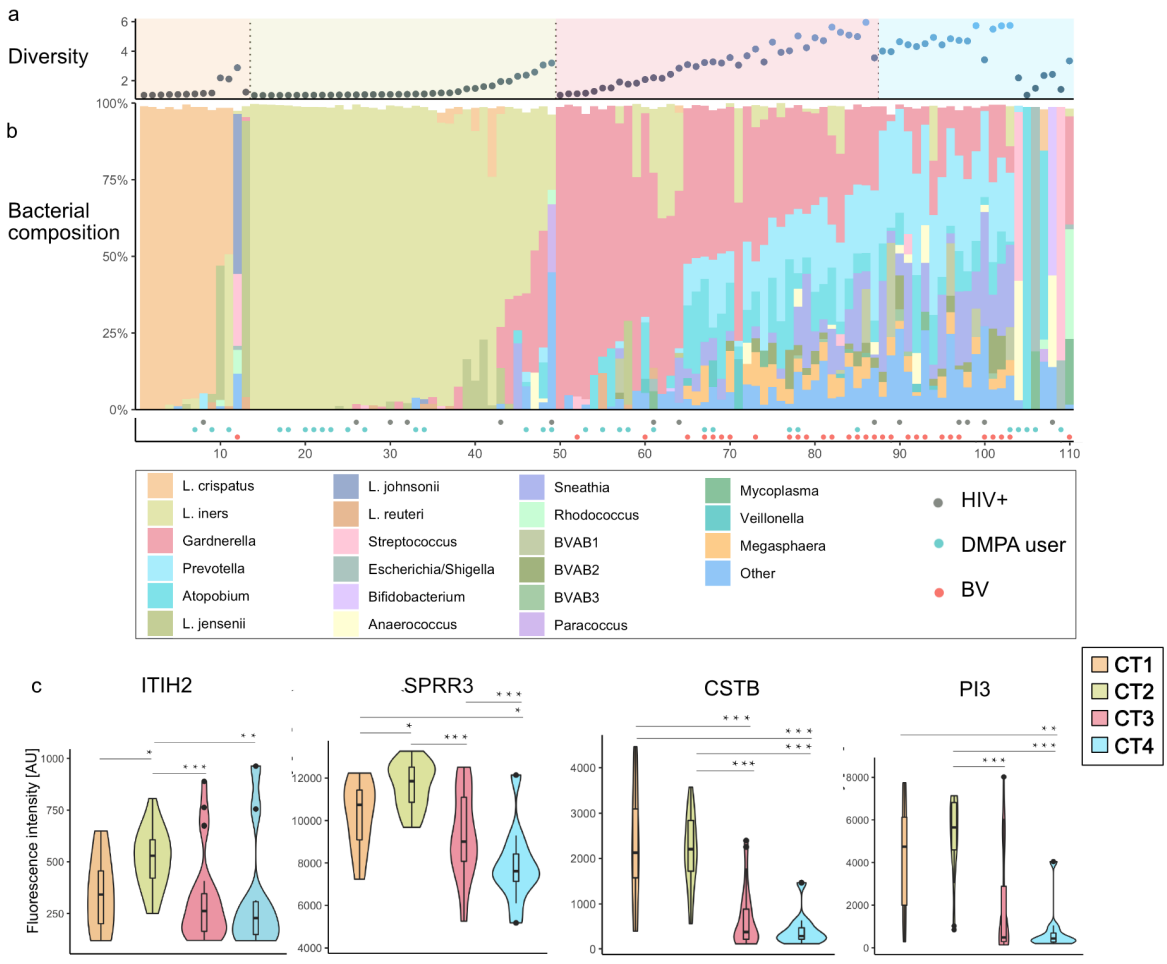

Figure 6. Microbial composition in Kenyan female sex workers and their protein profiles.

*a) Alpha diversity index measured by inverse Simpson index. b) Taxonomy representations, each bar represents one sample, dots below bars show HIV seropositive status (gray), depot medroxyprogesterone acetate (DMPA) users (turquoise) and/or diagnosed with bacterial vaginosis (BV) (red). c) Secreted protein measures in relative abundance values for ITIH2 and SPRR3 that differed between Lactobacillus non-iners (CT1) and Lactobacillus iners (CT2), and for CSTB and PI3 that showed higher levels in Lactobacillus groups (CT1 and CT2) compared to non-Lactobacillus groups (CT3 and CT4). Boxplots represent median, IQR, range within 1.5 x IQR (whiskers), outliers represented as dots. The violin represents the entire range of the dataset. * P-value < 0.05, ** P-value <0.01, *** P-value < 0.001. P-values calculated by Wilcoxon rank sum test. CSTB: cystatin B, PI3: elafin, SPRR3: small proline rich protein 3, ITIH2: inter-alpha-trypsin inhibitor heavy chain. AU: arbitrary units. CT: cervicotype.*

non-iners group (Figure 6c). This upregulation may indicate an induction of repair mechanisms in the *L. iners* group. Apart from these two proteins, the two *Lactobacilli* groups (CT1 and CT2) were associated with a similar protein response in relation to the two non-*Lactobacilli* groups (CT3 and CT4) for the majority of protein analytes. Both CT1 and CT2 groups (the CT2 group being the strong link) displayed higher levels of several anti-proteases (CSTB, CSTA, PI3, SPINK5) and epithelial barrier proteins (SPRR3, SPINK5, KRT1, TACSTD2) relative to the CT3 and CT4 groups. The main function of CSTA and CSTB is inhibition of the pro-inflammatory proteases Cathepsin B, I and S [150], and the presence in

cervicovaginal secretion samples of these antiproteases significantly correlated with natural HIV resistance [151,152]. PI3 (also known as Elafin) is secreted by epithelial cells and has an important anti-inflammatory role, as well as participating in the control of epithelial barrier integrity [153]. In addition, PI3 has an HIV-inhibitory effect *in vivo* and its presence in cervicovaginal secretion samples correlated to natural HIV resistance in women at high risk of HIV infection [151,154,155]. The function of the epithelial barrier protein SPRR3 has been defined in detail for skin epithelium where it confers mechanical resistance as well as elastic properties [156]. Since SPRR3 is localized in the corresponding upper layer of the ectocervical epithelium [123] it can be assumed that it exerts similar effects also in mucosal tissue. Higher abundance of SPRR3, SPINK5 and several keratins in the *Lactobacillus* dominant compared to the *Lactobacillus* non-dominant CT groups have been shown in another study [16], in agreement with our present results.

Furthermore, to investigate the microbiome effect on HIV target cell frequency and location we measured $CD4^+$ cells in the epithelium. The overall frequency of the $CD4^+$ cells in the epithelium was similar between all CT groups. Nonetheless, there was a significant difference in the distribution of $CD4^+$ cells between the *Gardnerella* group (CT3) and the *L. iners* group (CT2). The *Gardnerella* group had a higher proportion of $CD4^+$ cells in the parabasal layer relative to the *L. iners* group, and a lower proportion in the lower IM layer. Both groups showed similar thickness of each layer. *Gardnerella* is known to cause sloughing of superficial cells leading to the characteristic clue cells, and has been shown to hinder wound healing in *in vitro* models[67]. The redistribution of $CD4^+$ cells could be a sign of $CD4^+$ cells moving up towards the upper layers to combat the infection, leading to an influx of cells in the parabasal layer and a reduction of cells in the lower IM layer. This theory would be in line with our hypothesis from paper II that a more basal localization of $CD103^-CD8^+$ cells reflect a recent influx of cells. Our image analysis here also showed that *Gardnerella* had lower proportion of $CD4^+$ cells in the lower IM layer compared to both CT1 and CT4 groups.

This study does not lack limitations. Many of the women in the study had been prescribed antibiotic drugs during a visit two weeks prior to sampling, and some women stated that they were currently taking these drugs. Antibiotics influences the vaginal microbiome and will be accounted for in future analysis. Many of the women with BV diagnosis were prescribed metronidazole, it is unclear what effects this drug has on the microbiome in general. As we noted in paper III, DMPA use had a strong effect on the epithelial barrier and CD4 cell

localization. We could not reliably do a sensitivity analysis of DMPA use, since splitting the groups in DMPA users and controls would result in too few women in each CT group.

In summary; by integrating the results of the microbiome classification with *in situ* digital image analysis and protein profiling of the paired ectocervical tissue and CVL samples, respectively, we here demonstrated novel molecular mechanisms of how the different microbiome groups may affect the genital mucosa. Our *in situ* image data highlighted the epithelial stability of the *L. non-iners* group (CT1) and indicated a change in spatial localization of $CD4^+$ cells in the *Gardnerella* group (CT3). The image data did not show a clear grouping of the *Lactobacillus* dominant vs. the *non-Lactobacillus* dominant groups, in contrast to a clear such grouping for the secreted protein profiles. The *Lactobacillus* dominant groups displayed several proteins related to epithelial stability, anti-inflammation and HIV protection compared to the *non-Lactobacillus* dominant groups.

10 CONCLUDING REMARKS

In this thesis the integrity of the mucosal barrier, and effector- and HIV target cells therein, were analyzed using *in situ* digital image analysis, protein profiling and flow cytometry. We focused on characterizing fresh-frozen tissue samples to get a snap-shot of real-life spatial organization of cells and tissue structures.

In paper I we introduced image analysis as a refined tool for evaluation of microbicide safety. We confirmed that a promising microbicide candidate, Q-GRFT, had no negative effect on the rectal epithelium while causing a small, but probably biologically negligable, increase in $CD4^+$ cells. We also discovered potential effects of multiple biopsy sampling that should be considered when designing pre-clinical studies.

In paper II we were the first to characterize TRM cells in the ectocervix of healthy and HIV infected women. We observed that HIV infected women displayed an altered phenotype in that they had increased levels of $CD103^-CD8^+$ TRM cells compared to HIV un-infected women, and that this may be due to a recent influx of these effector cells that have not yet upregulated the CD103 retention molecule.

In paper III we revealed potential mechanisms behind an increased HIV risk in women taking the hormonal contraception DMPA. DMPA users had a thinner superficial epithelial layer, and more HIV target cell receptors that were located closer to the vaginal lumen.

In paper IV we discovered by *in situ* analysis that *Lactobacillus non-iners* communities had a more intact epithelium, and *Gardnerella* dominated communities had a different spatial localization of $CD4^+$ cells. Secreted protein profiles showed that both *Lactobacillus* groups had elevated levels of anti-inflammatory and epithelial barrier proteins compared to the non-*Lactobacillus* dominant groups.

11 FUTURE DIRECTIONS

Recent technical advances, increases in computer capacity and cross-scientific collaborations have shifted medical science into the multi-omics era. In this thesis we demonstrated the benefits of extracting the vast amount of information contained in tissue images, and suggest digital image analysis as an "-omics" of its own.

Further development using digital image analysis would include multiplex immunostainings to capture immune cell sub-types, e.g. the main mucosal HIV target cell $CD4^+CCR5^+CCR6^+$ T cell, and characterization of multiple cell types within the same tissue sections. This would enable a better understanding of the cross-talk between immune cells such as Langerhans cells and $CD4^+$ T cells as well as between $CD4^+$ and $CD8^+$ T cells, and thus potential HIV transmission routes. Applying distance measures between cells, and from cells to lumen or other vital structures would be easily implemented. Furthermore, applying unsupervised machine learning algorithms on the tissue images could reveal interesting patterns that are not noticeable by eye but may distinguish different clinically relevant effects. Deeper analysis of the shape and size of cell nuclei in the different epithelial layers could also give information on potential drug effects. Even though tissue variability poses challenges, improved automation would reduce the time spent and decrease the learning curve.

For paper I, the safety of Q-GRFT microbicide is now being tested in humans and the image analysis workflow will be used to assess these biopsy samples. Validation of the image analysis workflow in another sample cohort, including potential addition of markers as mentioned above, could lead to an established method for safety assessment of HIV microbicides.

In the field of TRM cells in paper II, further phenotypic characterization of TRM cells using multiplex immunostaining and single-cell RNA transcriptomics would deepen our knowledge on the different sub-types. Many studies of TRM cells have been conducted in mouse models, which needs further validation in human material. Learning how to specifically activate TRM cells is of importance for vaccine development. Also, understanding of the mechanism behind upregulation of CD103 and retention in and interaction with the epithelium would be important.

Paper III revealed increased and more superficial localization of HIV target cells and a thinner superficial layer in DMPA-using women. HIV risk of using DMPA has recently been

explored in the Evidence for Contraceptive Options in HIV Outcomes (ECHO) trial. A total of 7829 women were randomly assigned to either DMPA, levonorgestrel implant or cupper-bearing IUD. Although no statistically significant HIV risk between the groups were seen, DMPA and cupper IUD inferred 18-29 % increased HIV risk compared to levonorgestrel. This study is controversial[157–159] and the complaints include performance of statistical analyses and the lack of a control group using no contraceptive method. Further research on how to improve and strengthen the epithelium and especially the superficial layer could result in combinatorial solutions that reduce the DMPA-associated HIV risk. Explant tissue or organoid systems could be used to assess if the lack of superficial layer is related to a higher HIV infectivity. Potential combinatorial effects of microbiome composition and DMPA use would be of interest to study, although our current cohort is most likely too small for this purpose.

The introduction of sequencing of the vaginal microbiota has revealed new species and introduced a quick way of profiling different bacterial compositions. As the field moves forward, we learn that DNA extraction and the choice of 16S variable region may affect the results. There are strain-level differences in bacterial characteristics that also vary depending on the bacterial environment. To follow up on the results presented in paper IV, we aim to analyze RNA transcriptomics data sequenced from cervical biopsies from the same women at the same timepoint as the microbiome data. This would reveal microbiome footprints on the host cells transcription that may be relatable to the epithelial integrity data obtained with image analysis. Furthermore, an extended protein panel will be used to add another layer of information on the local microenvironment. Another experiment would be to set up an artificial system; cultured epithelia, organoids or explants, and add different bacteria, combinations of bacteria or CVL from women with a specific community type. Applying the same techniques; transcriptomics, proteomics and image analysis, would validate the changes discovered in the human samples. These systems are multicellular and many include both epithelial cells and immune cells, and could thus be suitable to demonstrate the effects. One caveat is however that they still lack many properties of a living organism. Future work correlating certain bacterial species to our image and protein data could also reveal new connections. A follow up analysis of interest would be to compare the women's BMI with the microbiome profile as well as image data, to investigate effects of weight on microbiome composition, epithelial thickness and integrity.

Moving from sequencing of the 16S variable regions to shotgun sequencing of the whole bacterial genome would allow detailed species and even strain level characterization. Studies show that BV-associated *Gardnerella* is more cytotoxic than non-BV associated *Gardnerella*, and that *L. iners* express different genes depending on bacterial environment. Also, there seem to be regional differences on strain-level, comparison of *L.iners* strains from our Kenyan cohort vs. e.g. a Swedish cohort may suggest why BV is more prevalent in certain regional areas. To take this concept to the next level, analysis of the bacterial metabolomics would give a deeper understanding of the analytes present in the genital mucosa. Similar metabolites can be produced by different bacterial species, and the metabolic landscape can have a different composition than the bacterial community composition. Zevin et al. showed that proteins from *Lactobacillus* and *G. vaginalis* comprised a larger portion of the soluble proteome load than their relative abundance as measured by 16S rRNA gene sequencing, suggesting that these bacteria dominate the metabolic landscape of the FGT[67].

Women's health has in general been understudied, both due to gender inequality issues but also due to ethical restrictions excluding women of child-bearing age to participate in clinical trials. The fact that HIV is now targeting more women than men highlights the importance of better understanding the female genital mucosa. A combination of different -omics techniques could increase our knowledge in genital health using RNA sequencing, proteomics, microbiome sequencing and as we highlight in this thesis, digital image analysis. Understanding the complex local immune-milieu would lead to improved interventions to reduce both STI, cancer and fertility problems.

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